



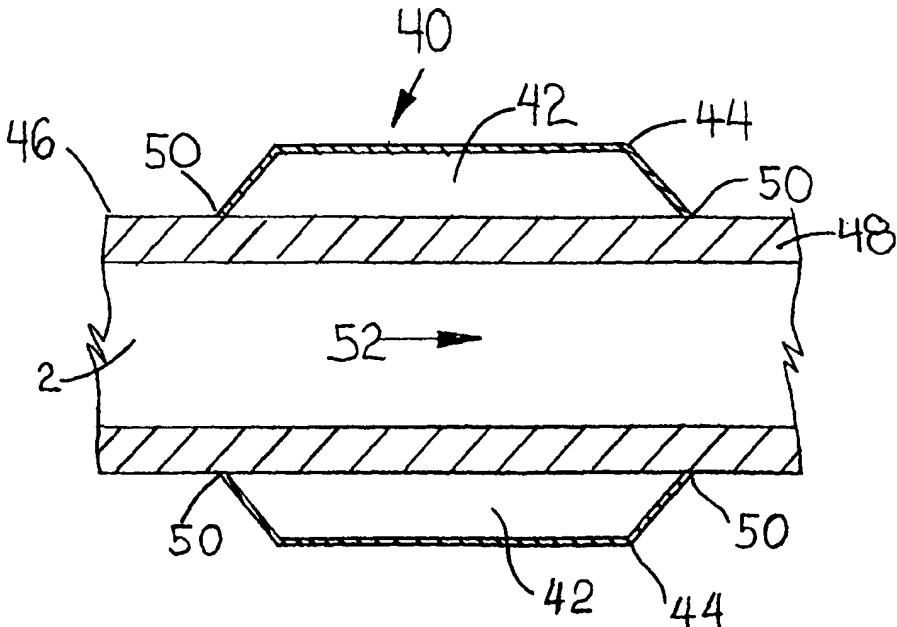
PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : <b>A61K 38/00</b>		A2	(11) International Publication Number: <b>WO 00/24412</b> (43) International Publication Date: 4 May 2000 (04.05.00)
(21) International Application Number: PCT/US99/24054			(72) Inventors: ALITALO, Kari; University of Helsinki, Haartman Institute, Molecular/Cancer Biology Laboratory, P.O. Box 21, (Haartmaninkatu 3), FIN-00014 Helsinki (FI). HILTUNEN, Mikko, O.; University of Kuopio, A.I. Virtanen Institute, P.O. Box 1627, FIN-70211 Kuopio (FI). JELTSCH, Markku, M.; University of Helsinki, Haartman Institute, Molecular/Cancer Biology Laboratory, P.O. Box 21, (Haartmaninkatu 3), FIN-00014 Helsinki (FI). ACHEN, Marc, G.; Ludwig Institute for Cancer Research, Melbourne Branch, P.O. Box Royal Melbourne Hospital, Parkville, VIC 3050 (AU).
(22) International Filing Date: 26 October 1999 (26.10.99)			(74) Agent: GASS, David, A.; Marshall, O'Toole, Gerstein, Murray & Borun, 6300 Sears Tower, 233 S. Wacker Drive, Chicago, IL 60606-6402 (US).
(30) Priority Data: 60/105,587 26 October 1998 (26.10.98) US			(81) Designated States: AU, CA, CN, JP, NO, NZ, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
(71) Applicants: LUDWIG INSTITUTE FOR CANCER RESEARCH [US/US]; 33rd floor, 605 Third Avenue, New York, NY 10158 (US). HELSINKI UNIVERSITY LICENSING LTD. OY [FI/FI]; Viikinkaari 6, FIN-00710 Helsinki (FI).			<b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
(71)(72) Applicant and Inventor: YLA-HERTTUALA, Seppo [FI/FI]; University of Kuopio, A.I. Virtanen Institute, P.O. Box 1627, FIN-70211 Kuopio (FI).			

(54) Title: USE OF VEGF-C OR VEGF-D GENE OR PROTEIN TO PREVENT RESTENOSIS



(57) Abstract

The present invention provides materials and methods for preventing stenosis or restenosis of a blood vessel using Vascular Endothelial Growth Factor C (VEGF-C) and/or Vascular Endothelial Growth Factor D (VEGF-D) genes or proteins.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## USE OF VEGF-C OR VEGF-D GENE OR PROTEIN TO PREVENT RESTENOSIS

5

### FIELD OF THE INVENTION

The present invention provides materials and methods to prevent stenosis and restenosis of blood vessels, and relates generally to the field of cardiovascular medicine.

### BACKGROUND OF THE INVENTION

10       Coronary artery disease constitutes a major cause of morbidity and mortality throughout the world, especially in the United States and Europe. Percutaneous transluminal coronary angioplasty (e.g., balloon angioplasty, with or without intracoronary stenting) is now a common and successful therapy for such disease, performed hundreds of thousands of times per year in the United States alone. However, 15       restenosis occurs in as many as one-third to one-half of such revascularization procedures, usually within six months of the angioplasty procedure. The economic cost of restenosis has been estimated at \$2 billion annually in the United States alone. [Feldman *et al.*, *Cardiovascular Research*, 32: 194-207 (1996), incorporated herein by reference.] Autopsy and atherectomy studies have identified intimal hyperplasia as the major histologic 20       component of restenotic lesions. [Cerek *et al.*, *Am. J. Cardiol.*, 68: 24C-33C (1991).]

Restenosis also remains a clinical concern in angioplasty that is performed in peripheral blood vessels. Likewise, stenosis is a clinical concern following transplantation of blood vessels (e.g., grafted veins and grafted artificial vessels) for cardiac bypass therapy or for treatment of peripheral ischemia or intermittent claudication, 25       for example (e.g., above-knee femoro-popliteal arterial bypass grafts).

Mazur *et al.*, *Texas Heart Institute Journal*, 21: 104-111 (1994) state that restenosis is primarily a response of the artery to the injury caused by percutaneous coronary angioplasty, which disrupts the intimal layer of endothelial cells and underlying

- 2 -

smooth muscle cells of the media. The authors state that multiple growth factors secreted by platelets, endothelial cells, macrophages, and smooth muscle cells are mechanistically involved in the restenosis process, and that proliferation of smooth muscle cells constitutes a critical pathogenetic feature. According to the authors, this smooth muscle cell  
5 proliferation has proven refractory to mechanical and pharmacologic therapy. More recently, others have called into question whether smooth muscle cell proliferation is of penultimate importance in restenosis. See Libby, *Circ. Res.*, 82: 404-406 (1998).

Narins *et al.*, *Circulation*, 97: 1298-1305 (1998) review the use of intracoronary stents and their benefits and limitations in preventing restenosis. Debbas *et*  
10 *al.*, *American Heart Journal*, 133: 460-468 (1997) discuss stenting within a stent to treat in-stent restenosis.

Chang & Leiden, *Semin. Intervent. Cardiol.*, 1: 185-193 (1996), incorporated herein by reference, review somatic gene therapy approaches to treat restenosis. Chang and Leiden teach that replication-deficient adenoviruses comprise a  
15 promising and safe vector system for gene therapy directed toward prevention of restenosis, because such viruses can efficiently infect a wide variety of cell types, including vascular smooth muscle cells; such viruses can be produced at high titers (e.g., 10<sup>10</sup>-10<sup>12</sup> plaque forming units per milliliter); such viruses can accommodate a transgene insert of, e.g., 7-9 kilobases (kb) in size; such viruses can be delivered percutaneously through  
20 standard catheters; and such viruses do not integrate into the host genome. Both Chang & Leiden and Feldman *et al.*, *supra*, also review cytotoxic and cytostatic gene therapy approaches, designed to kill or arrest proliferating vascular smooth muscle cells thought to be responsible for neointimal formations that characterize restenosis.

Riessen & Isner, *J. Am. Coll. Cardiol.*, 23:1234-1244 (1994), incorporated by reference, review devices for intravascular drug delivery and vectors for intravascular  
25 gene therapy.

Cerek *et al.*, *Am. J. Cardiol.*, 68: 24C-33C (1991) suggest prevention of restenosis by inhibiting growth-factor-mediated healing of arterial injury. Potential roles of platelet-derived growth factor (PDGF), thrombospondin, insulin-like growth factor 1  
30 (IGF-1), fibroblast growth factors (FGF's), transforming growth factor alpha (TGF- $\alpha$ ) and beta (TGF- $\beta$ ), epidermal growth factor (EGF) are discussed.

Isner & Asahara, International Patent Publication No. WO 98/19712, incorporated herein by reference, suggest treating injured blood vessels and accelerating reendothelialization following angioplasty by isolating a patient's endothelial progenitor cells and re-administering such cells to the patient. The authors suggest that the 5 effectiveness of using an angiogenesis-promoting growth factor, such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF), may be limited by the lack of endothelial cells on which the VEGF or bFGF will exert its effect.

Martin *et al.*, International Patent Publication No. WO 98/20027 suggest the use of VEGF gene or protein to treat or prevent stenosis or restenosis of a blood 10 vessel. The authors suggest that any beneficial effect of VEGF arises from a different mechanism of action than the mechanism underlying an activity of VEGF related to stimulating re-endothelialisation in cases where the endothelium has been damaged.

Callow *et al.*, *Growth Factors*, 10: 223-228 (1994) state that intravenous 15 injection of vascular permeability factor (a.k.a. VEGF) into rabbits that had been subjected to balloon angioplasty-induced endothelial denudation resulted in increased regeneration of endothelium compared to a control. The authors also stated that basic fibroblast growth factor (bFGF) is effective at promoting re-endothelialization, but that such re-endothelialization is accompanied by increases in neointimal lesion size.

Asahara *et al.*, *Circulation*, 94: 3291-3302 (December 15, 1996) state that 20 local, percutaneous catheter delivery of a CMV-human-VEGF<sub>165</sub> transgene achieved accelerated re-endothelialization in balloon-injured rabbits, and resulted in diminished intimal thickening. In a report by a related group of authors, Van Belle *et al.*, *J. Am. Coll. Cardiol.*, 29:1371-1379 (May, 1997) state that stent endothelialization was accelerated by 25 delivery of a CMV-human-VEGF<sub>165</sub> transgene and was accompanied by attenuation of intimal thickening.

Morishita *et al.*, *J. Atherosclerosis and Thrombosis*, 4(3): 128-134 (1998) state that hepatocyte growth factor (HGF) has a mitogenic activity on human endothelial cells more potent than VEGF, and hypothesized that HGF gene therapy may have potential therapeutic value for the treatment of cardiovascular diseases such as restenosis after 30 angioplasty. Morishita *et al.* also state that there is little knowledge about growth factors that stimulate only endothelial cells, but not vascular smooth muscle cells.

- 4 -

DeYoung & Dicke, *Circ. Res.*, 82: 306-313 (1998) state that VEGF gene delivery does not currently appear destined for application to human coronary restenosis, and that two independent studies suggest that VEGF delivery may actually worsen arterial intimal hyperplasia.

5 Brown *et al.*, U.S. Patent No. 5,795,898, suggest using an inhibitor of PDGF, FGF, EGF, or VEGF signaling to suppress accelerated atherogenesis involved in restenosis of coronary vessels or other arterial vessels following angioplasty.

The foregoing discussion demonstrates that a long-felt need continues to exist for improvements to angioplasty materials and/or methods, and/or for adjunct 10 therapies, to reduce instances of restenosis.

#### SUMMARY OF THE INVENTION

The present invention addresses long-felt needs in the field of medicine by providing materials and methods for the prevention of stenosis or restenosis in mammalian blood vessels.

15 For example, the invention provides a method of treating a mammalian subject to prevent stenosis or restenosis of a blood vessel, comprising the step of administering to a mammalian subject in need of treatment to prevent stenosis or restenosis of a blood vessel a composition comprising a polynucleotide, the polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor C 20 (VEGF-C) polypeptide. In a preferred embodiment, the subject is a human subject.

While it is contemplated that the VEGF-C polynucleotide could be administered purely as a prophylactic treatment to prevent stenosis, it is contemplated in a preferred embodiment that the polynucleotide be administered shortly before, and/or concurrently with, and/or shortly after a percutaneous transluminal coronary angioplasty 25 procedure, for the purpose of preventing restenosis of the subject vessel. In another preferred embodiment, the polynucleotide is administered before, during, and/or shortly after a bypass procedure (e.g., a coronary bypass procedure), to prevent stenosis or restenosis in or near the transplanted (grafted) vessel, especially stenosis at the location of the graft itself. In yet another embodiment, the polynucleotide is administered before, 30 during, or after a vascular transplantation in the vascular periphery that has been

- 5 -

performed to treat peripheral ischemia or intermittent claudication. By prevention of stenosis or restenosis is meant prophylactic treatment to reduce the amount/severity of, and/or substantially eliminate, the stenosis or restenosis that frequently occurs in such surgical procedures. The polynucleotide is included in the composition in an amount and 5 in a form effective to promote expression of a VEGF-C polypeptide in a blood vessel of the mammalian subject, thereby preventing stenosis or restenosis of the blood vessel.

In a preferred embodiment, the mammalian subject is a human subject. For example, the subject is a person suffering from coronary artery disease that has been identified by a cardiologist as a candidate who could benefit from a therapeutic balloon 10 angioplasty (with or without insertion of an intravascular stent) procedure or from a coronary bypass procedure. Practice of methods of the invention in other mammalian subjects, especially mammals that are conventionally used as models for demonstrating therapeutic efficacy in humans (e.g., primate, porcine, canine, or rabbit animals), also is contemplated.

15 For the practice of methods of the invention, the term "VEGF-C polypeptide" is intended to include any polypeptide that has a VEGF-C or VEGF-C analog amino acid sequence (as defined elsewhere herein in greater detail) and that possesses *in vivo* restenosis-reducing effects of human VEGF-C, which effects are demonstrated herein by way of example in a rabbit model. The term "VEGF-C polynucleotide" is intended to 20 include any polynucleotide (e.g., DNA or RNA, single- or double-stranded) comprising a nucleotide sequence that encodes a VEGF-C polypeptide. Due to the well-known degeneracy of the genetic code, there exist multiple VEGF-C polynucleotide sequences that encode any selected VEGF-C polypeptide.

For treatment of humans, VEGF-C polypeptides with an amino acid 25 sequence of a human VEGF-C are highly preferred, and polynucleotides comprising a nucleotide sequence of a human VEGF-C cDNA are highly preferred. By "human VEGF-C" is meant a polypeptide corresponding to a naturally occurring protein (prepro-protein, partially-processed protein, or fully-processed mature protein) encoded by any allele of the human VEGF-C gene, or a polypeptide comprising a biologically active fragment of a 30 naturally-occurring mature protein. By way of example, a human VEGF-C comprises a continuous portion of the amino acid sequence set forth in SEQ ID NO: 2 sufficient to

- 6 -

permit the polypeptide to bind and stimulate VEGFR-2 and/or VEGFR-3 phosphorylation in cells that express such receptors. A polypeptide comprising amino acids 131-211 of SEQ ID NO: 2 is specifically contemplated. For example, polypeptides having an amino acid sequence comprising a continuous portion of SEQ ID NO: 2, the continuous portion having, as its amino terminus, an amino acid selected from the group consisting of positions 30-131 of SEQ ID NO: 2, and having, as its carboxyl terminus, an amino acid selected from the group consisting of positions 211-419 of SEQ ID NO: 2 are contemplated. As explained elsewhere herein in greater detail, VEGF-C biological activities, especially those mediated through VEGFR-2, increase upon processing of both an amino-terminal and carboxyl-terminal pro-peptide. Thus, an amino terminus selected from the group consisting of positions 102-131 of SEQ ID NO: 2 is preferred, and an amino terminus selected from the group consisting of positions 103-113 of SEQ ID NO: 2 is highly preferred. Likewise, a carboxyl terminus selected from the group consisting of positions 211-227 of SEQ ID NO: 2 is preferred. As stated above, the term "human VEGF-C" also is intended to encompass polypeptides encoded by allelic variants of the human VEGF-C characterized by the sequences set forth in SEQ ID NOs: 1 & 2.

Moreover, since the therapeutic VEGF-C is to be administered as recombinant VEGF-C or indirectly via somatic gene therapy, it is within the skill in the art to make and use analogs of human VEGF-C (and polynucleotides that encode such analogs) wherein one or more amino acids have been added, deleted, or replaced with other amino acids, especially with conservative replacements, and wherein the anti-restenosis biological activity has been retained. Analogs that retain anti-restenosis VEGF-C biological activity are contemplated as VEGF-C polypeptides for use in the present invention. In a preferred embodiment, analogs having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 such modifications and that retain anti-restenosis VEGF-C biological activity are contemplated as VEGF-C polypeptides for use in the present invention. Polynucleotides encoding such analogs are generated using conventional PCR, site-directed mutagenesis, and chemical synthesis techniques.

Also contemplated as VEGF-C polypeptides are non-human mammalian or avian VEGF-C polypeptides and polynucleotides. By "mammalian VEGF-C" is meant a polypeptide corresponding to a naturally occurring protein (prepro-protein, partially-

processed protein, or fully-processed mature protein) encoded by any allele of a VEGF-C gene of any mammal, or a polypeptide comprising a biologically active fragment of a mature protein. The term "mammalian VEGF-C polypeptide" is intended to include analogs of mammalian VEGF-C's that possess the *in vivo* restenosis-reducing effects of 5 the mammalian VEGF-C. The fact that gene therapy using a transgene encoding human VEGF-C is effective to prevent restenosis in a rabbit model is evidence of the inter-species therapeutic efficacy of VEGF-C proteins.

Irrespective of which VEGF-C polypeptide is chosen, the VEGF-C polynucleotide preferably comprises a nucleotide sequence encoding a secretory signal 10 peptide fused in-frame with the VEGF-C polypeptide sequence. The secretory signal peptide directs secretion of the VEGF-C polypeptide by the cells that express the polynucleotide, and is cleaved by the cell from the secreted VEGF-C polypeptide. For example, the VEGF-C polynucleotide could encode the complete prepro-VEGF-C sequence set forth in SEQ ID NO: 2; or could encode the VEGF-C signal peptide fused in- 15 frame to a sequence encoding a fully-processed VEGF-C (e.g., amino acids 103-227 of SEQ ID NO: 2) or VEGF-C analog. Moreover, there is no requirement that the signal peptide be derived from VEGF-C. The signal peptide sequence can be that of another secreted protein, or can be a completely synthetic signal sequence effective to direct secretion in cells of the mammalian subject.

20 In one embodiment, the VEGF-C polynucleotide of the invention comprises a nucleotide sequence that will hybridize to a polynucleotide that is complementary to the human VEGF cDNA sequence specified in SEQ ID NO: 1 under the following exemplary stringent hybridization conditions: hybridization at 42°C in 50% formamide, 5X SSC, 20 mM Na<sub>2</sub>PO<sub>4</sub>, pH 6.8; and washing in 1X SSC at 55°C for 30 minutes; and wherein the 25 nucleotide sequence encodes a polypeptide that binds and stimulates human VEGFR-2 and/or VEGFR-3. It is understood that variation in these exemplary conditions occur based on the length and GC nucleotide content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining appropriate hybridization conditions. *See Sambrook et al., Molecular Cloning: A Laboratory Manual* (Second ed., 30 Cold Spring Harbor Laboratory Press, 1989) §§ 9.47-9.51.

- 8 -

In preferred embodiments, the VEGF-C polynucleotide further comprises additional sequences to facilitate the VEGF-C gene therapy. In one embodiment, a "naked" VEGF-C transgene (i.e., a transgene without a viral, liposomal, or other vector to facilitate transfection) is employed for gene therapy. In this embodiment, the VEGF-C polynucleotide preferably comprises a suitable promoter and/or enhancer sequence (e.g., cytomegalovirus promoter/enhancer [Lehner *et al.*, *J. Clin. Microbiol.*, 29:2494-2502 (1991); Boshart *et al.*, *Cell*, 41:521-530 (1985)]; Rous sarcoma virus promoter [Davis *et al.*, *Hum. Gene Ther.*, 4:151 (1993)]; Tie promoter [Korhonen *et al.*, *Blood*, 86(5): 1828-1835 (1995)]; or simian virus 40 promoter) for expression in the target mammalian cells, 5 the promoter being operatively linked upstream (i.e., 5') of the VEGF-C coding sequence. The VEGF-C polynucleotide also preferably further includes a suitable polyadenylation sequence (e.g., the SV40 or human growth hormone gene polyadenylation sequence) operably linked downstream (i.e., 3') of the VEGF-C coding sequence. The polynucleotide may further optionally comprise sequences whose only intended function is 10 to facilitate large-scale production of the vector, e.g., in bacteria, such as a bacterial origin of replication and a sequence encoding a selectable marker. However, in a preferred embodiment, such extraneous sequences are at least partially cleaved off prior to administration to humans according to methods of the invention. One can manufacture and administer such polynucleotides to achieve successful gene therapy using procedures 15 that have been described in the literature for other transgenes. See, e.g., Isner *et al.*, *Circulation*, 91: 2687-2692 (1995); and Isner *et al.*, *Human Gene Therapy*, 7: 989-1011 (1996); incorporated herein by reference in the entirety.

Any suitable vector may be used to introduce the VEGF-C transgene into the host. Exemplary vectors that have been described in the literature include replication-25 deficient retroviral vectors, including but not limited to lentivirus vectors [Kim *et al.*, *J. Virol.*, 72(1): 811-816 (1998); Kingsman & Johnson, *Scrip Magazine*, October, 1998, pp. 43-46.]; adeno-associated viral vectors [Gnatenko *et al.*, *J. Investig. Med.*, 45: 87-98 (1997)]; adenoviral vectors [See, e.g., U.S. Patent No. 5,792,453; Quantin *et al.*, *Proc. Natl. Acad. Sci. USA*, 89: 2581-2584 (1992); Stratford-Perricaudet *et al.*, *J. Clin. Invest.*, 30 90: 626-630 (1992); and Rosenfeld *et al.*, *Cell*, 68: 143-155 (1992)]; Lipofectin-mediated gene transfer (BRL); liposomal vectors [See, e.g., U.S. Patent No. 5,631,237 (Liposomes

- 9 -

comprising Sendai virus proteins)] ; and combinations thereof. All of the foregoing documents are incorporated herein by reference in the entirety. Replication-deficient adenoviral vectors constitute a preferred embodiment.

In embodiments employing a viral vector, preferred polynucleotides still 5 include a suitable promoter and polyadenylation sequence as described above. Moreover, it will be readily apparent that, in these embodiments, the polynucleotide further includes vector polynucleotide sequences (e.g., adenoviral polynucleotide sequences) operably connected to the sequence encoding a VEGF-C polypeptide.

Thus, in one embodiment the composition to be administered comprises a 10 vector, wherein the vector comprises the VEGF-C polynucleotide. In a preferred embodiment, the vector is an adenovirus vector. In a highly preferred embodiment, the adenovirus vector is replication-deficient, i.e., it cannot replicate in the mammalian subject due to deletion of essential viral-replication sequences from the adenoviral genome. For example, the inventors contemplate a method wherein the vector comprises a replication- 15 deficient adenovirus, the adenovirus comprising the VEGF-C polynucleotide operably connected to a promoter and flanked on either end by adenoviral polynucleotide sequences.

The composition to be administered according to methods of the invention 20 preferably comprises (in addition to the polynucleotide or vector) a pharmaceutically- acceptable carrier solution such as water, saline, phosphate-buffered saline, glucose, or other carriers conventionally used to deliver therapeutics intravascularly. Multi-gene therapy is also contemplated, in which case the composition optionally comprises both the VEGF-C polynucleotide/vector and another polynucleotide/vector selected to prevent restenosis. Exemplary candidate genes/vectors for co-transfection with VEGF-C 25 transgenes are described in the literature cited above, including genes encoding cytotoxic factors, cytostatic factors, endothelial growth factors, and smooth muscle cell growth/migration inhibitors. As described in greater detail below, a VEGF-D transgene is a preferred candidate for co-administration with the VEGF-C transgene. Co- administration of a VEGF transgene also is specifically contemplated.

30 The "administering" that is performed according to the present method may be performed using any medically-accepted means for introducing a therapeutic directly or

- 10 -

indirectly into the vasculature of a mammalian subject, including but not limited to injections; oral ingestion; intranasal or topical administration; and the like. In a preferred embodiment, administration of the composition comprising the VEGF-C polynucleotide is performed intravascularly, such as by intravenous, intra-arterial, or intracoronary arterial 5 injection.

In a highly preferred embodiment, the composition is administered locally, e.g., to the site of angioplasty or bypass. For example, the administering comprises a catheter-mediated transfer of the transgene-containing composition into a blood vessel of the mammalian subject, especially into a coronary artery of the mammalian subject.

10 Exemplary materials and methods for local delivery are reviewed in Lincoff *et al.*, *Circulation*, 90: 2070-2084 (1994); and Wilensky *et al.*, *Trends Cardiovasc. Med.*, 3:163-170 (1993), both incorporated herein by reference. For example, the composition is administered using infusion-perfusion balloon catheters (preferably microporous balloon catheters) such as those that have been described in the literature for intracoronary drug 15 infusions. See, e.g., U.S. Patent No. 5,713,860 (Intravascular Catheter with Infusion Array); U.S. Patent No. 5,087,244; U.S. Patent No. 5,653,689; and Wolinsky *et al.*, *J. Am. Coll. Cardiol.*, 15: 475-481 (1990) (Wolinsky Infusion Catheter); and Lambert *et al.*, *Coron. Artery Dis.*, 4: 469-475 (1993), all of which are incorporated herein by reference in their entirety. Use of such catheters for site-directed somatic cell gene therapy is 20 described, e.g., in Mazur *et al.*, *Texas Heart Institute Journal*, 21: 104-111 (1994), incorporated herein by reference. In an embodiment where the VEGF-C transgene is administered in an adenovirus vector, the vector is preferably administered in a pharmaceutically acceptable carrier at a titer of  $10^7$ - $10^{13}$  viral particles, and more preferably at a titer of  $10^9$ - $10^{11}$  viral particles. The adenoviral vector composition 25 preferably is infused over a period of 15 seconds to 30 minutes, more preferably 1 to 10 minutes.

For example, in patients with angina pectoris due to a single or multiple lesions in coronary arteries and for whom PTCA is prescribed on the basis of primary coronary angiogram findings, an exemplary protocol involves performing PTCA through a 30 7F guiding catheter according to standard clinical practice using the femoral approach. If an optimal result is not achieved with PTCA alone, then an endovascular stent also is

- 11 -

implanted. (A nonoptimal result is defined as residual stenosis of > 30 % of the luminal diameter according to a visual estimate, and B or C type dissection.) Arterial gene transfer at the site of balloon dilatation is performed with a replication-deficient adenoviral VEGF-C vector immediately after the angioplasty, but before stent implantation, using an infusion-perfusion balloon catheter. The size of the catheter will be selected to match the diameter of the artery as measured from the angiogram, varying, e.g., from 3.0 to 3.5F in diameter. The balloon is inflated to the optimal pressure and gene transfer is performed during a 10 minute infusion at the rate of 0.5 ml/min with virus titer of  $1.15 \times 10^{10}$ .

In another embodiment, intravascular administration with a gel-coated catheter is contemplated, as has been described in the literature to introduce other transgenes. See, e.g., U.S. Patent No. 5,674,192 (Catheter coated with tenaciously-adhered swellable hydrogel polymer); Riessen *et al.*, *Human Gene Therapy*, 4: 749-758 (1993); and Steg *et al.*, *Circulation*, 96: 408-411 (1997) and 90: 1648-1656 (1994); all incorporated herein by reference. Briefly, DNA in solution (e.g., the VEGF-C polynucleotide) is applied one or more times *ex vivo* to the surface of an inflated angioplasty catheter balloon coated with a hydrogel polymer (e.g., Slider with Hydroplus, Mansfield Boston Scientific Corp., Watertown, MA). The Hydroplus coating is a hydrophilic polyacrylic acid polymer that is cross-linked to the balloon to form a high molecular weight hydrogel tightly adhered to the balloon. The DNA covered hydrogel is permitted to dry before deflating the balloon. Re-inflation of the balloon intravascularly, during an angioplasty procedure, causes the transfer of the DNA to the vessel wall.

In yet another embodiment, an expandable elastic membrane or similar structure mounted to or integral with a balloon angioplasty catheter or stent is employed to deliver the VEGF-C transgene. See, e.g., U.S. Patent Nos. 5,707,385, 5,697,967, 5,700,286, 5,800,507, and 5,776,184, all incorporated by reference herein.

In another variation, the composition containing the VEGF-C transgene is administered extravascularly, e.g., using a device to surround or encapsulate a portion of vessel. See, e.g., International Patent Publication WO 98/20027, incorporated herein by reference, describing a collar that is placed around the outside of an artery (e.g., during a bypass procedure) to deliver a transgene to the arterial wall via a plasmid or liposome vector.

- 12 -

In still another variation, endothelial cells or endothelial progenitor cells are transfected *ex vivo* with the VEGF-C transgene, and the transfected cells as administered to the mammalian subject. Exemplary procedures for seeding a vascular graft with genetically modified endothelial cells are described in U.S. Patent No. 5,785,965, 5 incorporated herein by reference.

If the mammalian subject is receiving a vascular graft, the VEGF-C transgene-containing composition may be directly applied to the isolated vessel segment prior to its being grafted *in vivo*.

In another aspect, the invention provides a method of treating a mammalian 10 subject to prevent stenosis or restenosis of a blood vessel, comprising the step of administering to a mammalian subject in need of treatment to prevent stenosis or restenosis of a blood vessel a composition comprising a VEGF-C polypeptide, in an amount effective to prevent stenosis or restenosis of the blood vessel. In a preferred embodiment, the administering comprises implanting an intravascular stent in the mammalian subject, where 15 the stent is coated or impregnated with the composition. Exemplary materials for constructing a drug-coated or drug-impregnated stent are described in literature cited above and reviewed in Lincoff *et al.*, *Circulation*, 90: 2070-2084 (1994). In another preferred embodiment, the composition comprises microparticles composed of biodegradable polymers such as PGLA, non-degradable polymers, or biological polymers 20 (e.g., starch) which particles encapsulate or are impregnated by the VEGF-C polypeptide. Such particles are delivered to the intravascular wall using, e.g., an infusion angioplasty catheter. Other techniques for achieving locally sustained drug delivery are reviewed in Wilensky *et al.*, *Trends Cardovasc. Med.*, 3:163-170 (1993), incorporated herein by reference.

25 Administration via one or more intravenous injections subsequent to the angioplasty or bypass procedure also is contemplated. Localization of the VEGF-C polypeptides to the site of the procedure occurs due to expression of VEGF-C receptors on proliferating endothelial cells. Localization is further facilitated by recombinantly expressing the VEGF-C as a fusion polypeptide (e.g., fused to an apolipoprotein B-100 30 oligopeptide as described in Shih *et al.*, *Proc. Nat'l. Acad. Sci. USA*, 87:1436-1440

- 13 -

(1990). Co-administration of VEGF-C polynucleotides and VEGF-C polypeptides also is contemplated.

5 In yet another embodiment, the invention provides the use of a VEGF-C polynucleotide or VEGF-C polypeptide for the manufacture of a medicament for the treatment or prevention of stenosis or restenosis of a blood vessel.

In still another embodiment, the invention provides a method of treating a mammalian subject to prevent stenosis or restenosis of a blood vessel, comprising the step of administering to a mammalian subject in need of treatment to prevent stenosis or restenosis of a blood vessel a composition comprising a polynucleotide, the polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor D (VEGF-D) polypeptide. Such methods are practiced essentially as described herein with respect to VEGF-C-encoding polynucleotides, except that polynucleotides encoding VEGF-D are employed. A detailed description of the human VEGF-D gene and protein are provided in Achen, *et al.*, *Proc. Nat'l Acad. Sci. U.S.A.*, 95(2): 548-553 (1998);  
10 International Patent Publication No. WO 98/07832, published 26 February 1998; and in Genbank Accession No. AJ000185, all incorporated herein by reference. A cDNA and deduced amino acid sequence for prepro-VEGF-D is set forth herein in SEQ ID NOs: 3 and 4. Of course, due to the well-known degeneracy of the genetic code, there exist multiple VEGF-D encoding polynucleotide sequences, any of which may be employed  
15 according to the methods taught herein.

20 As described herein in detail with respect to VEGF-C, the use of polynucleotides that encode VEGF-D fragments, VEGF-D analogs, VEGF-D allelic and interspecies variants, and the like which possess *in vivo* anti-restenosis effects of human VEGF-D are all contemplated as being encompassed by the present invention.  
25 In yet another embodiment, the invention provides a method of treating a mammalian subject to prevent stenosis or restenosis of a blood vessel, comprising the step of administering to a mammalian subject in need of treatment to prevent stenosis or restenosis of a blood vessel a composition comprising a VEGF-D polypeptide, in an amount effective to prevent stenosis or restenosis of the blood vessel. Such methods are  
30 practiced essentially as described herein with respect to VEGF-C polypeptides.

In a related aspect, the invention provides materials and devices for practice of the above-described methods.

For example, the polynucleotides, polypeptides, vectors, compositions, and the like that are described for use in methods of the invention are themselves intended as 5 aspects of the invention.

Likewise, the invention also provides surgical devices that are used to treat circulatory disorders, such as intravascular (endovascular) stents, balloon catheters, infusion-perfusion catheters, extravascular collars, elastomeric membranes, and the like, which have been improved by coating with, impregnating with, adhering to, or 10 encapsulating within the device a composition comprising a VEGF-C polynucleotide, a VEGF-C polypeptide, a VEGF-D polynucleotide, and/or a VEGF-D polypeptide.

For example, in one embodiment, the invention provides an endovascular stent characterized by an improvement wherein the stent is coated or impregnated with a composition, the comprising at least one anti-restenosis agent selected from the group 15 consisting of VEGF-C polynucleotides, VEGF-C polypeptides, VEGF-D polynucleotides, and VEGF-D polypeptides. Exemplary stents that may be improved in this manner are described and depicted in U.S. Patent Nos. 5,800,507 and 5,697,967 (Medtronic, Inc., describing an intraluminal stent comprising fibrin and an elutable drug capable of providing a treatment of restenosis); U.S. Patent No. 5,776,184 (Medtronic, Inc., describing a stent 20 with a porous coating comprising a polymer and a therapeutic substance in a solid or solid/solution with the polymer); U.S. Patent No. 5,799,384 (Medtronic, Inc., describing a flexible, cylindrical, metal stent having a biocompatible polymeric surface to contact a body lumen); U.S. Patent Nos. 5,824,048 and 5,679,400; and U.S. Patent No. 5,779,729; all of which are specifically incorporated herein by reference in the entirety. Implantation 25 of such stents during conventional angioplasty techniques will result in less restenosis than implantation of conventional stents. In this sense, the biocompatibility of the stent is improved.

In another embodiment, the invention provides an extravascular collar for delivery of a therapeutic agent to a blood vessel, characterized by an improvement wherein 30 the collar is coated with or impregnated with or encapsulates a composition, the comprising at least one anti-restenosis agent selected from the group consisting of VEGF-

- 15 -

C polynucleotides, VEGF-C polypeptides, VEGF-D polynucleotides, and VEGF-D polypeptides. An exemplary collar to be improved in this manner is described and depicted in International Patent Publication WO 98/20027 (Eurogene, Ltd., collar comprising a body adopted to provide a seal around a vessel and to define a reservoir for holding an anti-restenosis pharmaceutical formulation), incorporated herein by reference.

5 In yet another embodiment, the invention provides a polymer film for wrapping a stent, characterized by an improvement wherein the film is coated with or impregnated with a composition, the comprising at least one anti-restenosis agent selected from the group consisting of VEGF-C polynucleotides, VEGF-C polypeptides, VEGF-D 10 polynucleotides, and VEGF-D polypeptides. An exemplary film to be improved in this manner is described and depicted in U.S. Patent Nos. 5,700,286 and 5,707,385 (Advanced Cardiovascular Systems, Inc., sheaths of bioabsorbable polymeric material coated or impregnated with a restenosis-preventing therapeutic agent and attachable to an endovascular stent).

15 Similarly, the invention includes kits which comprise compounds or compositions of the invention packaged in a manner which facilitates their use to practice methods of the invention. In a simplest embodiment, such a kit includes a compound or composition described herein as useful for practice of the invention (e.g., VEGF-C or VEGF-D polynucleotides or polypeptides), packaged in a container such as a sealed bottle 20 or vessel, with a label affixed to the container or included in the package that describes use of the compound or composition to practice the method of the invention. Preferably, the compound or composition is packaged in a unit dosage form. In another embodiment, a kit of the invention includes both a VEGF-C or VEGF-D polynucleotide or polypeptide composition packaged together with a physical device useful for implementing methods of 25 the invention, such as a stent, a catheter, an extravascular collar, a polymer film, or the like. In another embodiment, a kit of the invention includes both a VEGF-C or VEGF-D polynucleotide or polypeptide composition packaged together with a hydrogel polymer, or microparticle polymers, or other carriers described herein as useful for delivery of the VEGF-C/VEGF-D to the patient.

- 16 -

Additional features and variations of the invention will be apparent to those skilled in the art from the entirety of this application, and all such features are intended as aspects of the invention.

Likewise, features of the invention described herein can be re-combined 5 into additional embodiments that also are intended as aspects of the invention, irrespective of whether the combination of features is specifically mentioned above as an aspect or embodiment of the invention. Also, only such limitations which are described herein as critical to the invention should be viewed as such; variations of the invention lacking limitations which have not been described herein as critical are intended as aspects of the 10 invention.

In addition to the foregoing, the invention includes, as an additional aspect, all embodiments of the invention narrower in scope in any way than the variations specifically mentioned above. Although the applicant(s) invented the full scope of the 15 claims appended hereto, the claims appended hereto are not intended to encompass within their scope the prior art work of others. Therefore, in the event that statutory prior art within the scope of a claim is brought to the attention of the applicants by a Patent Office or other entity or individual, the applicant(s) reserve the right to exercise amendment rights under applicable patent laws to redefine the subject matter of such a claim to specifically exclude such statutory prior art or obvious variations of statutory prior art 20 from the scope of such a claim. Variations of the invention defined by such amended claims also are intended as aspects of the invention.

#### BRIEF DESCRIPTION OF THE DRAWING

Figure 1 depicts a cross-section of a blood vessel into which a drug delivery balloon catheter including a protective sheath has been inserted, the protective sheath 25 serving to cover the balloon during insertion and positioning.

Figure 2A depicts a perspective view of an expandable membrane having two layers that are spaced apart, prior to joining edges of the layers to each other.

Figure 2B depicts a perspective view of the membrane of Figure 2A that has been rolled into a tube and had opposite edges adjoined.

- 17 -

Figures 3A and 3B depict, in perspective (3A) and longitudinal cross-section (3B), schematic views of an extravascular collar surrounding a portion of a blood vessel.

Figure 4A depicts in cross-section a wire coated with a polymer or gel that  
5 can include (e.g., be impregnated with) a therapeutic composition.

Figure 4B depicts a perspective view of an intravascular stent formed from the wire of Figure 4A.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that when a gene encoding  
10 human Vascular Endothelial Growth Factor C (VEGF-C) is administered to a mammal that has suffered a vascular trauma, such as the trauma that can occur during conventional balloon angioplasty procedures, restenosis of the injured vessel is reduced or eliminated. An *in vivo* controlled experiment demonstrating the efficacy of a VEGF-C transgene to prevent restenosis is described in detail in Example 1. Example 2 provides a side-by-side  
15 comparative study demonstrating that the anti-restenosis effects of VEGF-C appear superior to the anti-restenosis effects of VEGF administered in a comparable manner.

The growth factor named Vascular Endothelial Growth Factor C (VEGF-C), as well as native human, non-human mammalian, and avian polynucleotide sequences encoding VEGF-C, and VEGF-C variants and analogs, have been described in detail in  
20 International Patent Application Number PCT/US98/01973, filed 02 February 1998 and published on 06 August 1998 as International Publication Number WO 98/33917; in Joukov *et al.*, *J. Biol. Chem.*, 273(12): 6599-6602 (1998); and in Joukov *et al.*, *EMBO J.*, 16(13): 3898-3911 (1997), all of which are incorporated herein by reference in the entirety. As explained therein in detail, human VEGF-C is initially produced in human  
25 cells as a prepro-VEGF-C polypeptide of 419 amino acids. A cDNA and deduced amino acid sequence for human prepro-VEGF-C are set forth in SEQ ID NOs: 1 and 2, respectively, and a cDNA encoding human VEGF-C has been deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209 (USA), pursuant to the provisions of the Budapest Treaty (Deposit date of 24 July 1995  
30 and ATCC Accession Number 97231). VEGF-C sequences from other species also have

- 18 -

been reported. See Genbank Accession Nos. MMU73620 (*Mus musculus*); and CCY15837 (*Coturnix coturnix*) for example, incorporated herein by reference.

The prepro-VEGF-C polypeptide is processed in multiple stages to produce a mature and most active VEGF-C polypeptide of about 21-23 kD (as assessed by SDS-PAGE under reducing conditions). Such processing includes cleavage of a signal peptide (SEQ ID NO: 2, residues 1-31); cleavage of a carboxyl-terminal peptide (corresponding approximately to amino acids 228-419 of SEQ ID NO: 2 and having a pattern of spaced cysteine residues reminiscent of a Balbiani ring 3 protein (BR3P) sequence [Dignam *et al.*, *Gene*, 88:133-40 (1990); Paulsson *et al.*, *J. Mol. Biol.*, 211:331-49 (1990)]) to produce a partially-processed form of about 29 kD; and cleavage (apparently extracellularly) of an amino-terminal peptide (corresponding approximately to amino acids 32-103 of SEQ ID NO: 2) to produce a fully-processed mature form of about 21-23 kD. Experimental evidence demonstrates that partially-processed forms of VEGF-C (e.g., the 29 kD form) are able to bind the Flt4 (VEGFR-3) receptor, whereas high affinity binding to VEGFR-2 occurs only with the fully processed forms of VEGF-C. It appears that VEGF-C polypeptides naturally associate as non-disulfide linked dimers.

Moreover, it has been demonstrated that amino acids 103-227 of SEQ ID NO: 2 are not all critical for maintaining VEGF-C functions. A polypeptide consisting of amino acids 113-213 (and lacking residues 103-112 and 214-227) of SEQ ID NO: 2 retains the ability to bind and stimulate VEGF-C receptors, and it is expected that a polypeptide spanning from about residue 131 to about residue 211 will retain VEGF-C biological activity. The cysteine residue at position 156 has been shown to be important for VEGFR-2 binding ability. However, VEGF-C  $\Delta C_{156}$  polypeptides (i.e., analogs that lack this cysteine due to deletion or substitution) remain potent activators of VEGFR-3. If the anti-restenosis effects of VEGF-C are mediated through VEGFR-3, then use of VEGF-C  $\Delta C_{156}$  polypeptides (and polynucleotides encoding them) is expected to provide anti-restenosis efficacy while minimizing VEGFR-2-mediated side-effects. The cysteine at position 165 of SEQ ID NO: 2 is essential for binding either receptor, whereas analogs lacking the cysteines at positions 83 or 137 compete with native VEGF-C for binding with both receptors and stimulate both receptors.

- 19 -

An alignment of human VEGF-C with VEGF-C from other species (performed using any generally accepted alignment algorithm) suggests additional residues wherein modifications can be introduced (e.g., insertions, substitutions, and/or deletions) without destroying VEGF-C biological activity. Any position at which aligned VEGF-C 5 polypeptides of two or more species have different amino acids, especially different amino acids with side chains of different chemical character, is a likely position susceptible to modification without concomitant elimination of function. An exemplary alignment of human, murine, and quail VEGF-C is set forth in Figure 5 of PCT/US98/01973.

Apart from the foregoing considerations, it will be understood that 10 innumerable conservative amino acid substitutions can be performed to a wildtype VEGF-C sequence which are likely to result in a polypeptide that retains VEGF-C biological activities, especially if the number of such substitutions is small. By "conservative amino acid substitution" is meant substitution of an amino acid with an amino acid having a side chain of a similar chemical character. Similar amino acids for making conservative 15 substitutions include those having an acidic side chain (glutamic acid, aspartic acid); a basic side chain (arginine, lysine, histidine); a polar amide side chain (glutamine, asparagine); a hydrophobic, aliphatic side chain (leucine, isoleucine, valine, alanine, glycine); an aromatic side chain (phenylalanine, tryptophan, tyrosine); a small side chain (glycine, alanine, serine, threonine, methionine); or an aliphatic hydroxyl side chain (serine, 20 threonine). Addition or deletion of one or a few internal amino acids without destroying VEGF-C biological activities also is contemplated.

Without intending to be limited to a particular theory, the mechanism 25 behind the efficacy of VEGF-C in preventing restenosis is believed to relate to the ability of VEGF-C to stimulate re-endothelialization of the injured vessel (and/or of the intravascular stent) without significant concomitant stimulation of smooth muscle proliferation in the vessel. VEGF-C also may inhibit smooth muscle cell proliferation. Accordingly, candidate VEGF-C analog polypeptides can be rapidly screened first for their ability to bind and stimulate autophosphorylation of known VEGF-C receptors (VEGFR-2 and VEGFR-3). Polypeptides that stimulate one or both known receptors are rapidly re-30 screened *in vitro* for their mitogenic and/or chemotactic activity against cultured capillary or arterial endothelial cells (e.g., as described in WO 98/33917). Polypeptides with

mitogenic and/or chemotactic activity are then screened *in vivo* as described herein for the ability to prevent restenosis. In this way, variants (analogs) of naturally occurring VEGF-C proteins are rapidly screened to determine whether or not the variants have the requisite biological activity to constitute "VEGF-C polypeptides" for use in the present invention.

5 The growth factor named Vascular Endothelial Growth Factor D (VEGF-D), as well as human sequences encoding VEGF-D, and VEGF-D variants and analogs, have been described in detail in International Patent Application Number PCT/US97/14696, filed 21 August 1997 and published on 26 February 1998 as International Publication Number WO 98/07832; and in Achen, *et al.*, *Proc. Nat'l Acad. Sci. U.S.A.*, 95(2): 548-553 (1998), both incorporated herein by reference in the entirety. 10 As explained therein in detail, human VEGF-D is initially produced in human cells as a prepro-VEGF-D polypeptide of 354 amino acids. A cDNA and deduced amino acid sequence for human prepro-VEGF-D are set forth in SEQ ID NOs: 3 and 4, respectively. VEGF-D sequences from other species also have been reported. See Genbank Accession 15 Nos. D89628 (*Mus musculus*); and AF014827 (*Rattus norvegicus*), for example, incorporated herein by reference.

The prepro-VEGF-D polypeptide has a putative signal peptide of 21 amino acids and is apparently proteolytically processed in a manner analogous to the processing of prepro-VEGF-C. A "recombinantly matured" VEGF-D lacking residues 1-92 and 202-20 354 of SEQ ID NO: 4 retains the ability to activate receptors VEGFR-2 and VEGFR-3, and appears to associate as non-covalently linked dimers. Thus, preferred VEGF-D polynucleotides include those polynucleotides that comprise a nucleotide sequence encoding amino acids 93-201 of SEQ ID NO: 4. The guidance provided above for introducing function-preserving modifications into VEGF-C polypeptides is also suitable 25 for introducing function-preserving modifications into VEGF-D polypeptides.

A therapeutic or prophylactic treatment of restenosis provided by the present invention involves administering to a mammalian subjection such as a human a composition comprising a VEGF-C or VEGF-D polynucleotide or polypeptide or combination thereof (sometimes generically referred to herein as a "VEGF-C or VEGF-D 30 therapeutic agent").

The "administering" may be performed using any medically-accepted means for introducing a therapeutic directly or indirectly into the vasculature of a mammalian subject, including but not limited to injections; oral ingestion; intranasal or topical administration; and the like. In a preferred embodiment, administration of the composition 5 comprising the VEGF-C or VEGF-D polynucleotide or polypeptide composition is performed intravascularly, such as by intravenous, intra-arterial, or intracoronary arterial injection.

In a highly preferred embodiment, the composition is administered locally, e.g., to the site of angioplasty or bypass. For example, the administering comprises a 10 catheter-mediated transfer of the therapeutic composition into a blood vessel of the mammalian subject, especially into a coronary artery of the mammalian subject. Exemplary materials and methods for local delivery are reviewed in Lincoff *et al.*, *Circulation*, 90: 2070-2084 (1994); and Wilensky *et al.*, *Trends Cardiovasc. Med.*, 3:163-170 (1993), both incorporated herein by reference. For example, the composition is 15 administered using infusion-perfusion balloon catheters (preferably microporous balloon catheters) such as those that have been described in the literature for intracoronary drug infusions. See, e.g., U.S. Patent No. 5,713,860 (Intravascular Catheter with Infusion Array); U.S. Patent No. 5,087,244; U.S. Patent No. 5,653,689; and Wolinsky *et al.*, *J. Am. Coll. Cardiol.*, 15: 475-481 (1990) (Wolinsky Infusion Catheter); and Lambert *et al.*, 20 *Coron. Artery Dis.*, 4: 469-475 (1993), all of which are incorporated herein by reference in their entirety. Use of such catheters for site-directed somatic cell gene therapy is described, e.g., in Mazur *et al.*, *Texas Heart Institute Journal*, 21; 104-111 (1994), incorporated herein by reference.

For example, in patients with angina pectoris due to a single or multiple 25 lesions in coronary arteries and for whom PTCA is prescribed on the basis of primary coronary angiogram findings, an exemplary protocol involves performing PTCA through a 7F guiding catheter according to standard clinical practice using the femoral approach. If an optimal result is not achieved with PTCA alone, then an endovascular stent also is implanted. (A nonoptimal result is defined as residual stenosis of > 30 % of the luminal 30 diameter according to a visual estimate, and B or C type dissection.) Arterial gene transfer at the site of balloon dilatation is performed immediately after the angioplasty, but before

- 22 -

stent implantation, using an infusion-perfusion balloon catheter. The size of the catheter will be selected to match the diameter of the artery as measured from the angiogram, varying, e.g., from 3.0 to 3.5F in diameter. The balloon is inflated to the optimal pressure and gene transfer is performed during a 10 minute infusion at the rate of 0.5 ml/min with 5 virus titer of  $1.15 \times 10^{10}$ .

In another embodiment, intravascular administration with a gel-coated catheter is contemplated, as has been described in the literature to introduce other transgenes. See, e.g., U.S. Patent No. 5,674,192 (Catheter coated with tenaciously-adhered swellable hydrogel polymer); Riessen *et al.*, *Human Gene Therapy*, 4: 749-758 10 (1993); and Steg *et al.*, *Circulation*, 96: 408-411 (1997) and 90: 1648-1656 (1994); all incorporated herein by reference. As shown in Figure 1, a catheter 10 is provided to which an inflatable balloon 12 is attached at a distal end. The balloon includes a swellable hydrogel polymer coating 14 capable of absorbing a solution comprising a therapeutic VEGF-C or VEGF-D therapeutic agent. Briefly, DNA in solution (e.g., the VEGF-C or 15 VEGF-D polynucleotide) is applied one or more times *ex vivo* to the surface of an inflated angioplasty catheter balloon coated with a hydrogel polymer (e.g., Slider with Hydroplus, Mansfield Boston Scientific Corp., Watertown, MA). The Hydroplus coating is a hydrophilic polyacrylic acid polymer that is cross-linked to the balloon to form a high molecular weight hydrogel tightly adhered to the balloon. The DNA covered hydrogel is 20 permitted to dry before deflating the balloon. Re-inflation of the balloon intravascularly, during an angioplasty procedure, causes the transfer of the DNA to the vessel wall. Thus, referring again to Figure 1, the catheter with attached, coated balloon is inserted into the lumen 16 of a blood vessel 18 while covered by a protective sheath 20 to minimize 25 exposure of the coated balloon to the blood prior to placement at the site of an occlusion. 22. When the instrument has been positioned at the treatment region, the protective sheath is drawn back or the catheter is moved forward to expose the balloon, which is inflated to compress the balloon (and thus the coating) into the vessel wall, causing transfer of the VEGF-C or VEGF-D therapeutic agent to the tissue, in a manner analogous to squeezing liquid from a compressed sponge or transferring wet paint to a surface by 30 contact.

- 23 -

In yet another embodiment, an expandable elastic membrane, film, or similar structure, mounted to or integral with a balloon angioplasty catheter or stent, is employed to deliver the VEGF-C or VEGF-D therapeutic agent. See, e.g., U.S. Patent Nos. 5,707,385, 5,697,967, 5,700,286, 5,800,507, and 5,776,184, all incorporated by reference herein. As shown in Figures 2A-2B, a single layer 30 or multi-layer 30, 32 sheet of elastic membrane material (Fig. 2A) is formed into a tubular structure 34 (Fig. 2B), e.g., by bringing together and adhering opposite edges of the sheet(s), e.g., in an overlapping or a abutting relationship. In this manner the elastomeric material may be wrapped around a catheter balloon or stent. A therapeutic VEGF-C or VEGF-D composition is combined with the membrane using any suitable means, including injection molding, coating, diffusion, and absorption techniques. In the multilayer embodiment depicted in the Figures, the edges of the two layers may be joined to form a fluid-tight seal. In a preferred embodiment, one layer of material is first processed by stretching the material and introducing a plurality of microscopic holes or slits 36. After the layers have been joined together, the sheet can be stretched and injected with the therapeutic VEGF-C/D composition through one of the holes or slits to fill the cavity that exists between the layers. The sheet is then relaxed, causing the holes to close and sealing the therapeutic composition between the layers until such time as the sheet is again stretched. This occurs, for example, at the time that an endovascular stent or balloon covered by the sheet is expanded within the lumen of a stenosed blood vessel. The expanding stent or balloon presses radially outward against the inner surface 38 of the tubular sheet covering, thus stretching the sheet, opening the holes, and delivering the therapeutic agent to the walls of the vessel.

In another variation, the composition containing the VEGF-C or VEGF-D therapeutic is administered extravascularly, e.g., using a device to surround or encapsulate a portion of vessel. See, e.g., International Patent Publication WO 98/20027, incorporated herein by reference, describing a collar that is placed around the outside of an artery (e.g., during a bypass procedure) to deliver a transgene to the arterial wall via a plasmid or liposome vector. As shown in Figures 3A and 3B, an extravascular collar 40 including a void space 42 defined by a wall 44 formed, e.g., of a biodegradable or biocompatible material. The collar touches the outer wall 46 of a blood vessel 48 at the collar's outer

- 24 -

extremities 50. Blood 52 flows through the lumen of the blood vessel. A longitudinal slit 54 in the flexible collar permits the collar to be deformed and placed around the vessel and then sealed using a conventional tissue glue, such as a thrombin glue.

In still another variation, endothelial cells or endothelial progenitor cells are 5 transfected *ex vivo* with the VEGF-C or VEGF-D transgene, and the transfected cells as administered to the mammalian subject. Exemplary procedures for seeding a vascular graft with genetically modified endothelial cells are described in U.S. Patent No. 5,785,965, incorporated herein by reference.

If the mammalian subject is receiving a vascular graft, the VEGF-C or 10 VEGF-D therapeutic composition may be directly applied to the isolated vessel segment prior to its being grafted *in vivo*.

In another preferred embodiment, the administering comprises implanting an intravascular stent in the mammalian subject, where the stent is coated or impregnated with the therapeutic VEGF-C/D gene/protein composition. Exemplary materials for 15 constructing a drug-coated or drug-impregnated stent are described in literature cited above and reviewed in Lincoff *et al.*, *Circulation*, 90: 2070-2084 (1994). As shown in Figure 4A and 4B, a metal or polymeric wire 70 for forming a stent is coated with a composition 72 such as a porous biocompatible polymer or gel that is impregnated with (or can be dipped in or otherwise easily coated immediately prior to use with) a VEGF-C 20 or VEGF-D therapeutic composition. The wire is coiled, woven, or otherwise formed into a stent 74 suitable for implantation into the lumen of a vessel using conventional materials and techniques, such as intravascular angioplasty catheterization. Exemplary stents that may be improved in this manner are described and depicted in U.S. Patent Nos. 5,800,507 and 5,697,967 (Medtronic, Inc., describing an intraluminal stent comprising fibrin and an 25 elutable drug capable of providing a treatment of restenosis); U.S. Patent No. 5,776,184 (Medtronic, Inc., describing a stent with a porous coating comprising a polymer and a therapeutic substance in a solid or solid/solution with the polymer); U.S. Patent No. 5,799,384 (Medtronic, Inc., describing a flexible, cylindrical, metal stent having a biocompatible polymeric surface to contact a body lumen); U.S. Patent Nos. 5,824,048 30 and 5,679,400; and U.S. Patent No. 5,779,729; all of which are specifically incorporated herein by reference in the entirety. Implantation of such stents during conventional

- 25 -

angioplasty techniques will result in less restenosis than implantation of conventional stents. In this sense, the biocompatibility of the stent is improved.

In another preferred embodiment, the composition comprises microparticles composed of biodegradable polymers such as PGLA, non-degradable polymers, or 5 biological polymers (e.g., starch) which particles encapsulate or are impregnated by the VEGF-C or VEGF-C polypeptide/polynucleotide. Such particles are delivered to the intravascular wall using, e.g., an infusion angioplasty catheter. Other techniques for achieving locally sustained drug delivery are reviewed in Wilensky *et al.*, *Trends Cardovasc. Med.*, 3:163-170 (1993), incorporated herein by reference.

10 Administration via one or more intravenous injections subsequent to the angioplasty or bypass procedure also is contemplated. Localization of the VEGF-C or VEGF-D polypeptides to the site of the procedure occurs due to expression of VEGF-C/D receptors on proliferating endothelial cells. Localization is further facilitated by recombinantly expressing the VEGF-C or VEGF-D as a fusion polypeptide (e.g., fused to 15 an apolipoprotein B-100 oligopeptide as described in Shih *et al.*, *Proc. Nat'l. Acad. Sci. USA*, 87:1436-1440 (1990).

The pharmaceutical efficacy of VEGF-C polynucleotides, VEGF-C polypeptides, VEGF-D polynucleotides, and VEGF-D polypeptides to prevent stenosis or restenosis of a blood vessel is demonstrated *in vivo*, e.g., using procedures such as those 20 described in the following examples, some of which are prophetic. The examples assist in further describing the invention, but are not intended in any way to limit the scope of the invention.

#### Example 1

##### Use of adenovirus-mediated VEGF-C gene transfer to prevent restenosis

25 The following experiments, performed *in vivo* in a rabbit restenosis model, demonstrate the efficacy of adenovirus-mediated intravascular VEGF-C gene transfer for the prevention of post-angioplasty restenosis.

A. Materials and Methods

## 1. Adenoviral constructs.

An adenovirus plasmid containing a cDNA encoding the complete human prepro-VEGF-C open reading frame operably linked to a cytomegalovirus (CMV) promoter and human growth hormone polyadenylation signal sequence was constructed as follows. A DNA fragment comprising a CMV promoter sequence was prepared by digesting the pcDNA3.1+ vector (Invitrogen) with *Sal* I and filling-in the 5' overhangs with the Klenow enzyme. The CMV promoter (nucleotides 5431-911) was excised from the vector with *Hind* III and isolated. A full-length human VEGF-C cDNA containing the 1997 bp sequence specified in SEQ ID NO: 1 (as well as less than 50 bases of additional non-coding and polylinker sequence) was excised from a VEGF-C pREP7 expression vector [described in WO 98/33917] with *Hind* III and *Xho* I and isolated. A human growth hormone polyadenylation signal (~ 860 bp) was excised from an  $\alpha$ MHC vector with *Sal* I and *Bam* HI. The CMV promoter, VEGF-C cDNA, and hGH polyadenylation signal fragments were simultaneously ligated into a *Bam* HI and *Eco* RV-digested pCRII vector. The ligated CMV promoter and VEGF-C cDNA is shown in SEQ ID NO: 17. The resulting construct was opened with *Bgl* II and partially-digested with *Bam* HI. The full transcriptional unit was ligated into *Bgl* II-opened pAdBglII vector. This construct [designated pAdBglII VEGF-C] was then used to create recombinant adenovirus containing the CMV-VEGF-C-hGH transcriptional unit, using standard homologous recombination techniques. [Barr *et al.*, *Gene Ther.*, 1: 51-58 (1994).] Replication-deficient E1-E3 deleted adenoviruses were produced in 293 cells and concentrated by ultracentrifugation using techniques known in the literature. [See, e.g., Barr *et al.* (1994).] A control plasmid comprising the *lacZ* gene operably linked to the same promoter was also used. [Laitinen M. *et al.*, *Hum. Gene Ther.*, 9: 1481-1486 (1998).] The *lacZ* adenovirus had a nuclear targeted signal, to direct the  $\beta$ -galactosidase expression to the nucleus. Replication-deficient E1-E3 deleted adenoviruses were produced in 293 cells and concentrated by ultracentrifugation (Barr *et al.*, 1994). The adenoviral preparations were analyzed for the absence of helper viruses and bacteriological contaminants.

## 2. Animal model.

New Zealand White rabbits were employed for the gene transfer study. A first group of rabbits was fed a 0.25 % cholesterol diet for two weeks, then subjected to balloon denudation of the aorta, then subjected three days later to the adenovirus-mediated gene transfer. A second group of rabbits was only subjected to the gene transfer. Animals were sacrificed 2 or 4 weeks after the gene transfer. The number of experimental (VEGF-C) and control (*lacZ*) animals in both study groups was 6.

In the first group of rabbits, the whole aorta, beginning from the tip of the arch, was denuded using a 4.0 F arterial embolectomy catheter (Sorin Biomedical, Irvine, CA). The catheter was introduced via the right iliac artery up to the aortic arch and inflated, and the aorta was denuded twice.

## 3. Gene transfer.

The gene transfer was performed using a 3.0 F channel balloon local drug delivery catheter (Boston Scientific Corp., Maple Grove, MA). Using fluoroscopical control, the balloon catheter was positioned caudal to the left renal artery, in a segment free of side branches, via a 5 F percutaneous introducer sheath (Arrow International, Reading, PA) in the right carotid artery and inflated to 6 ATM with a mixture of contrast media and saline. The anatomical location of the balloon catheter was determined by measuring its distance from the aortic orifice of the left renal artery. Virus titer of 1.15 x 10<sup>10</sup> plaque forming units (pfu) was administered to each animal in a final volume of 2 ml (0.9 % NaCl), and the gene transfer was performed at 6 ATM pressure for 10 minutes (0.2 ml / min). In the second study group the animals had only gene transfer and they were sacrificed 2 weeks after the gene transfer. The number of animals in each study group (0.9 % NaCl only; *lacZ* gene transfer; and VEGF-C gene transfer) was 3. All studies were approved by Experimental Animal Committee of the University of Kuopio in Finland.

## 4. Histology.

Three hours before sacrifice, the animals were injected intravenously with 50 mg of BrdU dissolved in 40 % ethanol. After the sacrifice, the aortic segment where the gene transfer had been performed was removed, flushed gently with saline, and divided into five equal segments. The proximal segment was snap frozen in liquid nitrogen and stored at -70°C. The next segment was immersion-fixed in 4 % paraformaldehyde / 15 %

- 28 -

sucrose (pH 7.4) for 4 hours, rinsed in 15 % sucrose (pH 7.4) overnight, and embedded in paraffin. The medial segment was immersion-fixed in 4 % paraformaldehyde / phosphate buffered saline (PBS) (pH 7.4) for 10 minutes, rinsed 2 hours in PBS, embedded in OCT compound (Miles), and stored at -70°C. The fourth segment was immersion-fixed in 70 % 5 ethanol overnight and embedded in paraffin. The distal segment was directly stained for  $\beta$ -galactosidase activity in X-GAL staining solution at +37°C for 16 hours, immersion-fixed in 4 % paraformaldehyde / 15 % sucrose (pH 7.4) for 4 hours, rinsed in 15 % sucrose overnight, and embedded in paraffin. Paraffin sections were used for immunocytochemical 10 detection of smooth muscle cells (SMC), macrophages, and endothelium. Gene transfer efficiency was evaluated using X-GAL staining of OCT-embedded tissues. BrdU-positive 15 cells were detected according to manufacturer's instructions. Morphometry was performed using haematoxylin-eosin stained paraffin sections using image analysis software. Measurements were taken independently by two observers from multiple sections, without knowledge of the origin of the sections. Intima/media (I/M) ratio was used as a parameter for intimal thickening.

B. Results.

Histological analysis of the balloon-denuded mice revealed that the *lacZ*-transfected control group had an I/M ratio of 0.61 two weeks after the gene transfer, which represented a statistically significant difference ( $p < 0.05$ ) from the VEGF-C-transfected groups (I/M ratio of 0.40). The tendency that VEGF-C group had a smaller 20 I/M ratio persisted at 4 weeks time point after the gene transfer.

In the second group of rabbits that were subjected only to gene transfer to the vessel wall (without endothelial denudation), the I / M ratio in the *lacZ* group was 0.3, compared to 0.15 for the VEGF-C group. This difference, too, represented a statistically 25 significant ( $p < 0.05$ ) inhibition in neointima formation in VEGF-C group.

The BrdU labeling will permit analysis of smooth muscle cell proliferation in VEGF-C-transfected versus control (*lacZ*) animals. SMC proliferation is expected to be reduced in the VEGF-C-transfected population.

The foregoing data demonstrate that VEGF-C gene transfer significantly 30 reduced intimal thickening at two weeks time point after aortic denudation and after vessel wall damage caused by the gene transfer catheter without balloon denudation. These data

indicate a therapeutic utility for VEGF-C gene transfer for the prevention of post-angioplasty restenosis.

### Example 2

#### Comparative Example Demonstrating that Anti-Restenosis

5                   Effects of VEGF-C Appear Superior to Those of VEGF.

The following experiments demonstrate the efficacy of adenovirus-mediated intravascular VEGF and VEGF-C gene transfer for the prevention of post-angioplasty restenosis, and demonstrates that VEGF-C appeared to provide a superior therapeutic efficacy compared to VEGF.

10                  A.    Materials and Methods

1.                 Adenoviral constructs.

VEGF (murine VEGF-A<sub>164</sub>; SEQ ID NO: 18) adenovirus was constructed using the same promoter as the VEGF-C construct, and following similar procedure as described in Example 1. The VEGF-A<sub>164</sub> adenoviral construct was produced in 293T cells and concentrated essentially as described in Example 1, and analyzed to be free of helper virus, lipopolysaccharides, and bacterial contaminants.

15                 2.    Animal model.

20                 Sixty three New Zealand White rabbits were divided into two major groups, the first having 0.25% cholesterol diet for two weeks and balloon denudation of the aorta before gene transfer, and the second group having only the gene transfer. Gene transfer was performed in the first group of rabbits three days after denudation, and the animals were sacrificed 2 or 4 weeks after the gene transfer. Number of rabbits in each study group (*lacZ*, VEGF, and VEGF-C) at both time points was 6. In the second study group, the rabbits had only the gene transfer, without cholesterol diet or balloon denudation, and were sacrificed 2 or 4 weeks after the gene transfer. The number of rabbits in each study group (0.9% saline, *lacZ*, VEGF, and VEGF-C) was 3.

25                 3.    Gene transfer.

Gene transfer was performed according to the procedure described in Example 1.

- 30 -

#### 4. Histology

Histology was performed essentially as described in Example 1 with the following modifications: SMC were detected using HHF35 (DAKO, 1:50 dilution), macrophages were detected using RAM-11 (DAKO, 1:50 dilution), endothelium was 5 detected using CD31 (DAKO, 1:50 dilution), and T cells were detected using MCA 805 (DAKO, 1:100 dilution). Controls for immunostainings included incubations with class- and species matched immunoglobulins and incubations where primary antibodies were omitted. Morphometry and image analysis were performed using Image-Pro Plus™ software and an Olympus AX70 microscope (Olympus Optical, Japan). Statistical 10 analyses were performed using the ANOVA and modified *t*-test. *P*<0.05 was considered statistically significant.

#### B. Results

Histological analysis of the balloon-denuded rabbit aorta shows intimal thickening and SMC proliferation. Two weeks after gene transfer, the *lacZ* control group 15 had the highest I/M ratio ( $0.57 \pm 0.04$ ) whereas VEGF-C ( $0.38 \pm 0.02$ ) and VEGF ( $0.49 \pm 0.17$ ) groups showed decreased intimal thickening. The difference in I/M ratios between *lacZ* and VEGF-C groups was significant (*P*<0.05), whereas those between *lacZ* and VEGF groups were not statistically significant, at the two-week time point. The tendency 20 that both VEGF and VEGF-C groups had smaller I/M ratios persisted at the four week time point when the I/M ratio was  $0.73 \pm 0.16$ ,  $0.44 \pm 0.14$ , and  $0.63 \pm 0.21$  for the *lacZ*, VEGF-C, and VEGF groups, respectively. Hematoxylin-eosin and immunostainings of the transfected arteries indicate that intimal thickening in all arteries was composed predominantly of SMC.

Use of adenoviral vectors can lead to immunological and inflammatory 25 responses, partly because high titer adenovirus induces expression of NF $\kappa$ B and activates a CTL response. However, no signs of inflammation nor foam cell accumulation were detected as judged by macrophage and T-cell immunostainings. In addition, human clinical gene therapy grade viruses were used together with short exposure times in the transfected arteries, which may also help explain the absence of severe inflammatory reactions in this 30 study.

The percentage of proliferating cells was analyzed using BrdU labeling. No

significant differences were seen, although the VEGF-C group tended to have a lower proliferation rate, consistent with the observation that VEGF-C transduced arteries had smaller I/M ratios at both time points. Two weeks after balloon denudation, the percentage of proliferating cells was  $1.8 \pm 0.4$ ,  $2.2 \pm 0.7$ , and  $1.2 \pm 0.0$  for the *lacZ*, VEGF, and VEGF-C groups, respectively, and after four weeks, the percentage of proliferating cells was  $0.3 \pm 0.1$ ,  $1.2 \pm 0.5$ , and  $0.3 \pm 0.1$  for the *lacZ*, VEGF, and VEGF-C groups, respectively. Endothelial regrowth was analyzed by measuring the length of intact endothelium from histological sections. No significant differences were found between the study groups.

The potential of adenovirus to cause damage to the vessel wall and neointima formation was tested by performing high-titer adenovirus gene transfer to intact abdominal aorta of rabbits without balloon-denudation. Control rabbits were treated in the same way with 0.9% saline. The positioning of the gene transfer catheter caused some internal elastic lamina damage and moderate induction of neointima formation after the procedure. At the two-week time point, the I/M ratio in the *lacZ* group was  $0.24 \pm 0.06$ , in the control group  $0.28 \pm 0.05$ , in the VEGF-C group  $0.18 \pm 0.07$ , and in the VEGF group  $0.15 \pm 0.03$ . At the four-week time point the *lacZ* group had an I/M ratio of  $0.22 \pm 0.13$ , the VEGF-C group  $0.13 \pm 0.03$ , and the VEGF group  $0.23 \pm 0.11$ .

This study shows a beneficial therapeutic effect of intravascular adenovirus-mediated VEGF-C gene transfer on the vessel wall after balloon injury, and also compares VEGF-C and VEGF adenovirus-mediated gene transfer for the prevention of neointima formation. Although different receptor binding profiles of VEGF-C and VEGF might have led to different biological effects in the vessel wall, both VEGFs reduced intimal thickening two weeks after gene transfer. Thus, both VEGFs are potential candidates for vascular gene therapy of ischemic atherosclerotic diseases. However, according to this experiment, VEGF-C appears to prevent restenosis more effectively than VEGF in this model system. The superior ability of VEGF-C to prevent restenosis, as compared to VEGF, could be due to expression or activity of VEGFR-3 which is a receptor for VEGF-C and VEGF-D, but not for VEGF. Alternatively, the apparent superiority may be attributable to a restenosis-promoting effect of VEGF mediated through VEGFR-1 or due to differential ligand effects (VEGF-C versus VEGF) mediated thru the common receptor VEGFR-2,

which is reportedly expressed in vascular smooth muscle cells. [See Grosskreutz *et al.*, *Microvasc. Res.*, 58(2): 128-136 (September, 1999).]

### Example 3

#### Expression of transfected VEGFs in the aortic wall

5       Using the aortic segments from the same experimental animals described in Example 2, mRNA expression of *lacZ*, VEGF-C and VEGF (murine VEGF-A<sub>164</sub>) was analyzed in aortic tissue after gene transfer. Total RNA was extracted from transfected aortic segments using Trizol Reagent (Gibco-BRL), and 2 µg of RNA was used for cDNA synthesis. Primers for *lacZ*, VEGF-C and VEGF were designed to distinguish between 10 endogenous and transduced genes by selecting the 5' primers from the CMV promoter and the 3' primers from the coding regions.

For *lacZ* amplification, primers were: 5' primer 5'-

15       TTGGAGGCCTAGGCTTTGC-3' (SEQ ID NO: 5) and 3' primer 5'-  
ATACTGTCGTCGTCCCCTCA-3' (SEQ ID NO: 6). The first PCR cycle was an initial incubation at 96°C for 4 minutes followed by 80°C for 3 minutes during which the DNA polymerase was added. This was followed by 30 cycles, each consisting of 94°C for 45 seconds, 58°C for 45 seconds, and 72°C for 50 seconds, followed by a final extension of 72°C for 5 minutes. 5 µl of the first PCR product was used for the second PCR with 5' primer 5'-GGTAGAAGACCCCAAGGACTT-3'(SEQ ID NO: 7) and 3' primer 5'-  
20       CGCCATTGCCATTCAAG-3' (SEQ ID NO: 8). The first PCR cycle was an initial incubation at 96°C for 3 minutes followed by 80°C for 3 minutes followed by 32 cycles, each consisting of 94°C for 60 seconds, 58°C for 15 seconds, and 72°C for 90 seconds, followed by a final extension of 72°C for 5 minutes.

For VEGF-C amplification, primers were: 5' primer 5'-

25       CTGCTTACTGGCTTATCG-3' (SEQ ID NO: 9) and 3' primer 5'-  
CCTGTTCTCTGTTATGTTGC-3' (SEQ ID NO: 10). The first PCR cycle was an initial incubation at 96°C for 4 minutes followed by 80°C for 3 minutes during which the DNA polymerase was added. This was followed by 39 cycles each consisting of 94°C for 30 seconds, 56°C for 40 seconds, and 72°C for 90 seconds, followed by a final extension of 30 72°C for 5 minutes. 5 µl of the first PCR product was used for the second PCR with 5'

- 33 -

primer 5'-TCTCCAAAAAGCTACACCG-3' (SEQ ID NO: 11) and 3' primer 5'-CAAGTGCATGGTGGAAAGG-3' (SEQ ID NO: 12). The first PCR cycle was an initial incubation at 96°C for 3 minutes followed by 80°C for 3 minutes followed by 39 cycles each consisting of 94°C for 60 seconds, 57°C for 30 seconds, and 72°C for 90 seconds, 5 followed by a final extension of 72°C for 5 minutes.

For VEGF amplification, primers were: 5' primer 5'-TCGATCCATGAACTTCTGC-3' (SEQ ID NO: 13) and 3' primer 5'-TTCGTTAACTCAAGCTGCC-3' (SEQ ID NO: 14). The first PCR cycle was an initial incubation at 96°C for 4 minutes followed by 80°C for 3 minutes, followed by 39 cycles 10 each consisting of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 90 seconds, followed by a final extension of 72°C for 5 minutes. 5 µl of the first PCR product was used for the second PCR with 5' primer 5'-GACCCTGGCTTACTGCTG-3' (SEQ ID NO: 15) and 3' primer 5'-GGAACATTACACGTCTGCG-3' (SEQ ID NO: 16). The first PCR cycle was an initial incubation at 96°C for 3 minutes followed by 80°C for 3 15 minutes followed by 39 cycles each consisting of 94°C for 60 seconds, 54°C for 30 seconds, and 72°C for 90 seconds, followed by a final extension of 72°C for 5 minutes.

The mRNA of *lacZ*, VEGF-C and VEGF was detected in aortic wall tissue up to four weeks after gene transfer.

Gene transfer efficiency was evaluated by assaying *lacZ* expression, 20 analyzed by X-Gal staining for β-galactosidase activity, in OCT embedded tissue sections. Transfection efficiency was 1.1% ± 0.5 and 0.3% ± 0.1, two and four weeks respectively, after intravascular catheter-mediated gene transfer.

#### Example 4

##### Expression of VEGF receptors in the aortic wall

25 Using the experimental animals described in Example 2, VEGFR-1, VEGFR-2, and VEGFR-3 expression in aortic tissue was analyzed by immunostainings and *in situ* hybridization. Immunohistochemistry was performed using clone sc-316 (Santa Cruz Biotechnology, 1:50 dilution) to detect VEGFR-1, clone sc-6251 (Santa Cruz Biotechnology, 1:500 dilution) to detect VEGFR-2, and clone sc-637 (Santa Cruz Biotechnology, 1:300 dilution) to detect VEGFR-3. Controls for immunostainings 30

included incubations with class- and species matched immunoglobulins and incubations where primary antibodies were omitted. *In situ* hybridization of VEGF receptor mRNAs was carried out using <sup>33</sup>P-UTP labeled riboprobes. Expression of all receptors was localized to endothelium. VEGFR-2 was also expressed in neointimal SMCs.

5

Example 5Use of naked VEGF-C transgene therapy to prevent restenosis

The procedures described in Example 1 or 2 are repeated, with the following modifications. Instead of using an adenovirus vector for delivery of the VEGF-C transgene, a mammalian expression vector is constructed for direct gene transfer (of 10 naked plasmid DNA). The VEGF-C coding sequence is operably linked to a suitable promoter, such as the CMV promoter, and preferably linked to a suitable polyadenylation sequence, such as the human growth hormone polyadenylation sequence. Exemplary VEGF-C vectors can be modeled from vectors that have been described in the literature to perform vector-free gene transfer for other growth factors, by substituting a VEGF-C 15 coding sequence for a VEGF coding sequence. [See, e.g., Isner *et al.*, *Circulation*, 91: 2687-2692 (1995); and Isner *et al.*, *Human Gene Therapy*, 7: 989-1011 (1996), incorporated herein by reference.] vector. A similar construct comprising a *lacZ* gene is used as a control.

A Hydrogel-coated balloon catheter (Boston Scientific) is used to deliver 20 the VEGF-C transgene essentially as described in Asahara *et al.*, *Circulation*, 94: 3291-3302 (December 15, 1996), incorporated herein by reference. Briefly, an angioplasty balloon is prepared *ex vivo* by advancing the deflated balloon completely through a teflon protective sheath (Boston Scientific). The balloon is inflated and a conventional pipette is used to apply the transgene construct (e.g., 50 - 5000 µg transgene DNA in a saline 25 solution) to the Hydrogel polymer coating the external surface of the inflated balloon. After the transgene solution has dried, the balloon is deflated, withdrawn into the protective sheath, and re-inflated to minimize blood flow across the balloon surface until the balloon is properly positioned in the target artery.

Intima/media (I/M) ratio is again used as a parameter for intimal thickening. 30 Reduced I/M ratio in animals treated with the VEGF-C transgene-coated balloon catheter

- 35 -

is considered indicative of therapeutic efficacy. As described in Example 2, comparison of the therapeutic efficacy of VEGF-C gene transfer with other therapies, such as VEGF gene transfer, can be conducted in parallel.

Example 6

5      Use of VEGF-C gene therapy to prevent restenosis following angioplasty with stent

The procedures described in the preceding examples are repeated with the modification that initial balloon angioplasty is accompanied by implantation of a coronary stent using conventional procedures. The VEGF-C transgene is delivered concurrently or immediately before or after stent implantation essentially as described in the preceding examples. Increased quantities (e.g., two-fold to ten-fold) of the transgene (compared to angioplasty without stent) and increased transfection time may be desirable, as described in Van Belle *et al.*, *J. Am. Coll. Cardiol.*, 29:1371-1379 (May, 1997), incorporated by reference herein. Decreased neointimal thickening and/or decreased thrombotic occlusion in the VEGF-C gene-treated animals versus control animals treated with a marker gene is 15 considered evidence of the efficacy of the VEGF-C gene therapy.

Example 7

Use of an extravascular collar to reduce vascular stenosis.

An inert silicone collar such as described in International Patent Publication No. WO 98/20027 is surgically implanted around the carotid arteries of New Zealand 20 White Rabbits. The collar acts as an irritation agent that will induce intimal thickening, and contains a reservoir suitable for local delivery of a VEGF-C transgene or protein pharmaceutical formulation. Gene transfer, using the VEGF-C adenovirus construct or control construct described in Example 1 is initiated five days later by injecting  $10^8$ - $10^{11}$  pfu into the collar. Animals are sacrificed 14 or 28 days later and histological 25 examinations are performed as described in Example 1. Intima/media thickness ratio [Yla-Herttuala *et al.*, *Arteriosclerosis*, 6: 230-236 (1986)] is used as an indicia of stenosis. Reduced I/M ratio in the VEGF-C-transfected rabbits, as compared to the *lacZ* control rabbits, indicates therapeutic efficacy of VEGF-C gene transfer for preventing arterial stenosis.

Example 8Use of VEGF-C polypeptides to reduce or prevent restenosis

The procedures described in Example 1 are repeated except, instead of treating the test animals with an adenovirus containing a VEGF-C transgene or *lacZ* control, the animals are treated with a composition comprising a VEGF-C polypeptide in a pharmaceutically acceptable carrier (e.g., isotonic saline with serum albumin), or with carrier solution alone as a control. Test animals receive either 10, 100, 250, 500, 1000, or 5000 µg of a VEGF-C polypeptide via intra-arterial infusion, e.g., as described in Example 1. A second group of animals additionally receive an injection of the VEGF-C polypeptide 10 days later. The animals are sacrificed and histological examination performed as described in Example 1. Reduced I/M ratio in the VEGF-C-treated animals versus control animals provides evidence of the therapeutic efficacy of VEGF-C polypeptide treatment. Repetition of the experiment using various sustained-release VEGF-C formulations and materials as described above is expected to further enhance the therapeutic efficacy of the 15 VEGF-C polypeptide. Moreover, a treatment regimen comprising the simultaneous administration of VEGF-C protein (to provide immediate therapy to the target vessel) with a VEGF-C transgene (to provide sustained therapy for several days or weeks) is specifically contemplated as a variation of the invention.

Example 9Anti-stenosis/anti-restenosis activity of VEGF-D

The procedures described in the preceding examples are repeated using a composition comprising a VEGF-D polynucleotide or VEGF-D polypeptide in lieu of the VEGF-C polynucleotide/polypeptide, to demonstrate the ability of VEGF-D to prevent stenosis or restenosis of a blood vessel.

- 37 -

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those in the art, all of which are intended as aspects of the present invention. Accordingly, only such limitations as appear in the claims should be placed on the invention.

## CLAIMS

1. A method of treating a mammalian subject to prevent stenosis or restenosis of a blood vessel, comprising the step of:  
administering to a mammalian subject in need of treatment to prevent  
5 stenosis or restenosis of a blood vessel a composition comprising at least one anti-restenosis agent selected from the group consisting of a polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor C (VEGF-C) polypeptide, a polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor D (VEGF-D) polypeptide, a VEGF-C polypeptide, and a VEGF-D polypeptide,  
10 thereby preventing stenosis or restenosis of said blood vessel.
2. A method according to claim 1 wherein said mammalian subject is human.
3. A method according to claim 1 or 2 wherein said composition comprises a polynucleotide that comprises a nucleotide sequence encoding a mammalian VEGF-C polypeptide.
- 15 4. A method according to any of claims 1-3 wherein said composition comprises a polynucleotide that comprises a nucleotide sequence that encodes a human VEGF-C polypeptide.
5. A method according to claim 4 wherein said VEGF-C polypeptide comprises an amino acid sequence comprising a continuous portion of SEQ ID NO: 2, said  
20 continuous portion having, as its amino terminus, an amino acid selected from the group consisting of positions 30-131 of SEQ ID NO: 2, and having, as its carboxyl terminus, an amino acid selected from the group consisting of positions 211 to 419 of SEQ ID NO: 2.

- 39 -

6. A method according to claim 5 wherein said polynucleotide further comprises a nucleotide sequence encoding a secretory signal peptide, and wherein the sequence encoding the secretory signal peptide is connected in-frame with the sequence that encodes the VEGF-C polypeptide

5 7. A method according to claim 6 wherein said polynucleotide lacks a nucleotide sequence encoding amino acids 228-419 of SEQ ID NO: 2.

8. A method according to claim 6 or 7 wherein said polynucleotide lacks a nucleotide sequence encoding amino acids 32-102 of SEQ ID NO: 2.

9. A method according to any one of claims 6-8 wherein the polynucleotide  
10 further comprises a promoter sequence operably connected to the sequence that encodes the secretory signal sequence and VEGF-C polypeptide, wherein the promoter sequence promotes transcription of the sequence that encodes the secretory signal sequence and the VEGF-C polypeptide in cells of the mammalian subject.

10. A method according to claim 9 wherein the polynucleotide further  
15 comprises a polyadenylation sequence operably connected to the sequence that encodes the VEGF-C polypeptide.

11. A method according to any one of claims 3-10 wherein the composition comprises a gene therapy vector, said gene therapy vector comprising said polynucleotide.

12. A method according to claim 11 wherein said vector comprises a  
20 replication-deficient adenovirus, said adenovirus comprising the polynucleotide operably connected to a promoter and flanked by adenoviral polynucleotide sequences.

13. A method according to any one of claims 1-12 wherein the composition further comprises a pharmaceutically acceptable carrier.

- 40 -

14. A method according to any one of claims 1-13 wherein said administering comprises at least one intravascular injection of said composition.

15. A method according to any one of claims 1-13 wherein said administering comprises a catheter-mediated transfer of said composition into a blood vessel 5 of the mammalian subject.

16. A method according to claim 15 wherein said catheter-mediated gene transfer comprises introducing a catheter into a coronary artery of the mammalian subject, and releasing the composition into the coronary artery.

17. A method according to any one of claims 1-16 wherein said 10 administering is conducted in said human concurrently with a percutaneous transluminal coronary angioplasty.

18. A method according to claim 1 wherein the composition comprises a VEGF-C polypeptide, in an amount effective to prevent stenosis or restenosis of said blood vessel.

15 19. A method according to claim 18 wherein said administering comprises implanting an intravascular stent in said mammalian subject, and wherein the stent is coated or impregnated with the composition.

20. A treatment to prevent stenosis or restenosis of a blood vessel in a human, comprising delivering a replication-deficient adenovirus vector to the vessel, said 20 vector comprising a polynucleotide encoding at least one of polypeptide selected from a VEGF-C polypeptide and a VEGF-D polypeptide, and further comprising a promoter sequence to promote expression of the at least one polypeptide in cells of the blood vessel, thereby preventing stenosis or restenosis of the blood vessel.

- 41 -

21. A method according to claim 20 wherein the polynucleotide encodes a VEGF-C polypeptide.

22. A medical device designed to contact a surface of a blood vessel in the course of surgery to treat stenosis of the blood vessel, the device characterized by an improvement comprising integrating into the device a composition effective to prevent restenosis, said composition comprising at least one anti-restenosis agent selected from the group consisting of a VEGF-C polynucleotide, a VEGF-C polypeptide, a VEGF-D polynucleotide, and a VEGF-D polypeptide.

23. The medical device of claim 22, wherein the device is selected from the group consisting of intravascular stents, intravascular catheters, extravascular collars, elastomeric membranes adapted to cover a surface of an intravascular stent or catheter, and combinations thereof.

24. A medical device according to claim 22 comprising an endovascular stent having an outer surface for contacting a surface of a blood vessel, and a composition on said surface, said composition comprising at least one anti-restenosis agent selected from the group consisting of a VEGF-C polynucleotide, a VEGF-C polypeptide, a VEGF-D polynucleotide, and a VEGF-D polypeptide.

25. A medical device according to claim 22 comprising a catheter having an outer surface for contacting a surface of a blood vessel, and a composition on said surface, said composition comprising at least one member selected from the group consisting of a VEGF-C polynucleotide, a VEGF-C polypeptide, a VEGF-D polynucleotide, and a VEGF-D polypeptide.

- 42 -

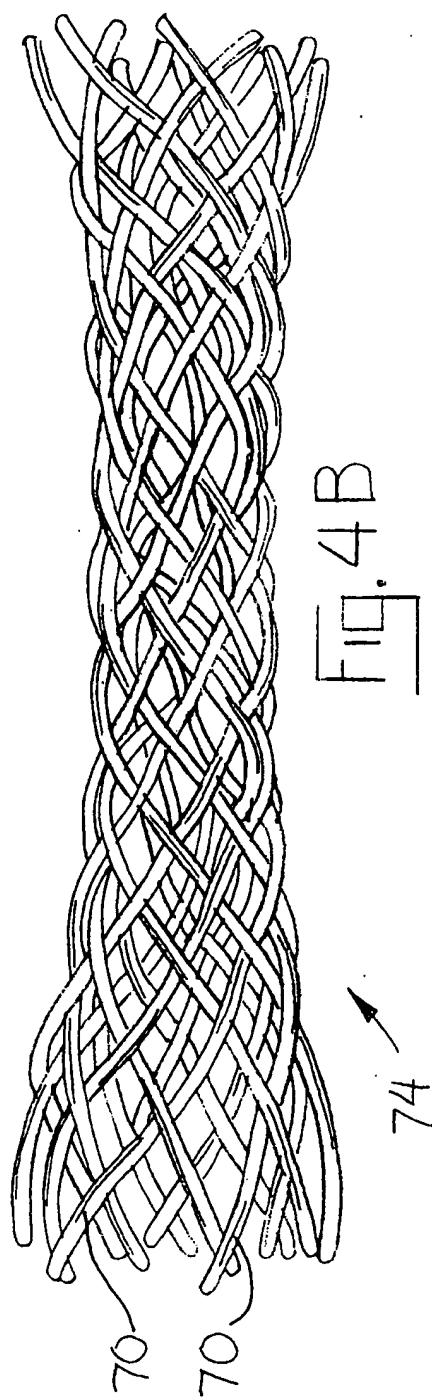
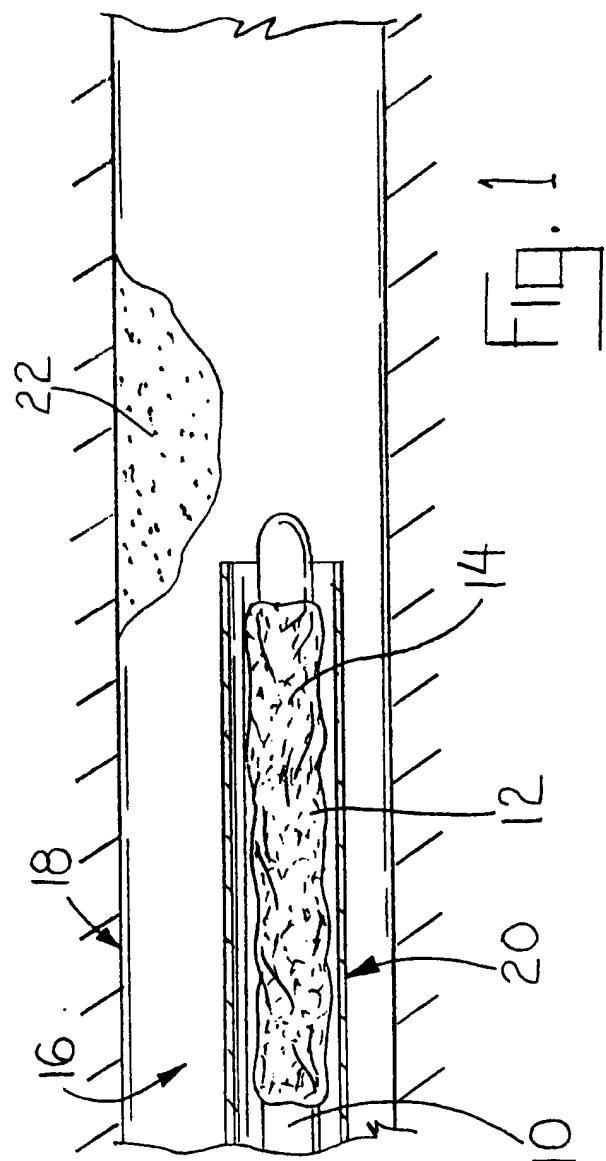
26. A medical device according to claim 22 comprising a balloon catheter having a void for holding a therapeutic agent for delivery to the interior of a blood vessel, and a composition contained in the void, the composition comprising at least one anti-restenosis agent selected from the group consisting of a VEGF-C polynucleotide, a VEGF-  
5 C polypeptide, a VEGF-D polynucleotide, and a VEGF-D polypeptide.

27. A kit for treating restenosis comprising a container holding at least one anti-restenosis agent selected from the group consisting of a VEGF-C polynucleotide, a VEGF-C polypeptide, a VEGF-D polynucleotide, and a VEGF-D polypeptide; and a label attached to or packaged with the container, the label describing use of the compound for  
10 prevention of restenosis of a blood vessel.

28. A kit according to claim 27, further comprising a medical device selected from the group consisting of: intravascular stents, intravascular catheters, extravascular collars, and membranes adapted to cover a surface of an intravascular stent or catheter.

15 29. The use of a composition for the manufacture of a medicament for the treatment or prevention of stenosis or restenosis of a blood vessel, said composition comprising at least one anti-restenosis agent selected from the group consisting of a polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor C (VEGF-C) polypeptide, a polynucleotide comprising a nucleotide sequence that  
20 encodes a vascular endothelial growth factor D (VEGF-D) polypeptide, a VEGF-C polypeptide, and a VEGF-D polypeptide.

1/4



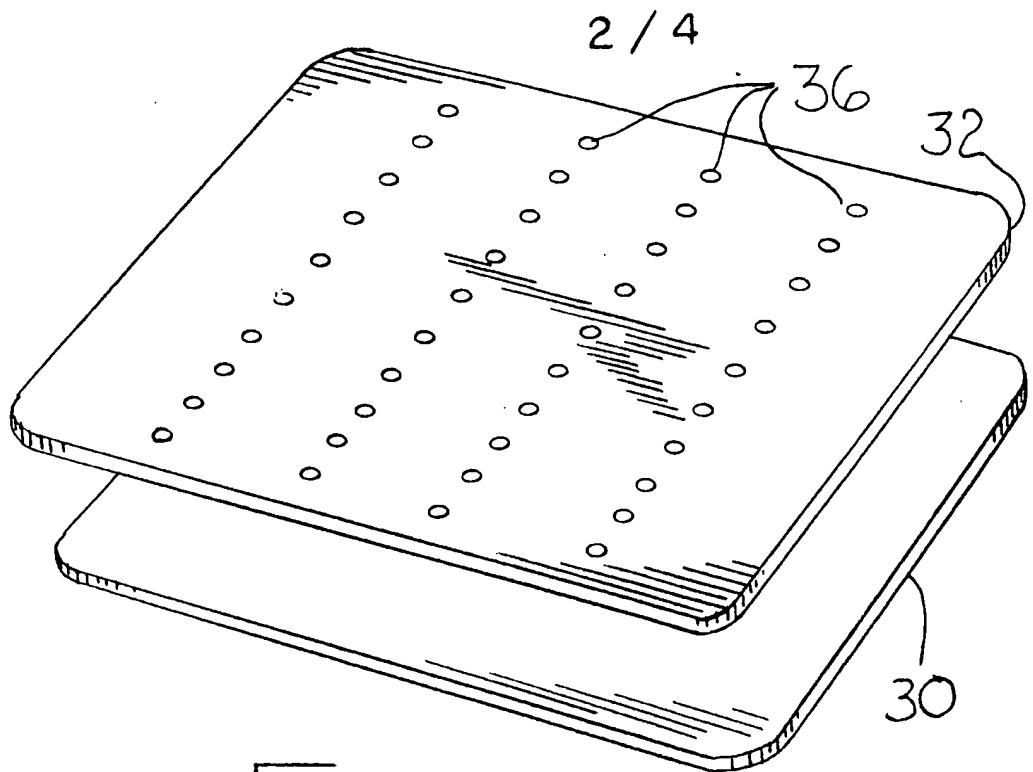


FIG. 2A

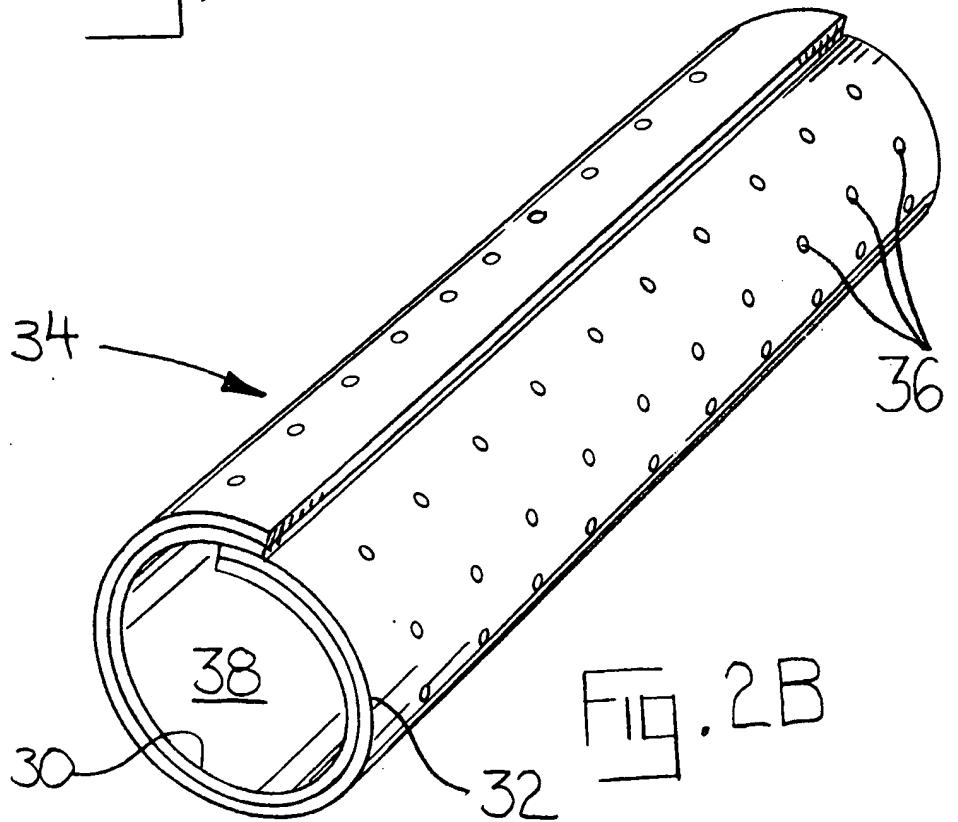
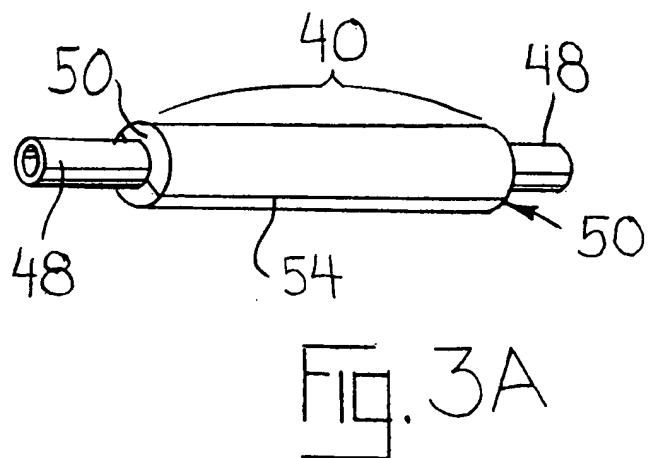
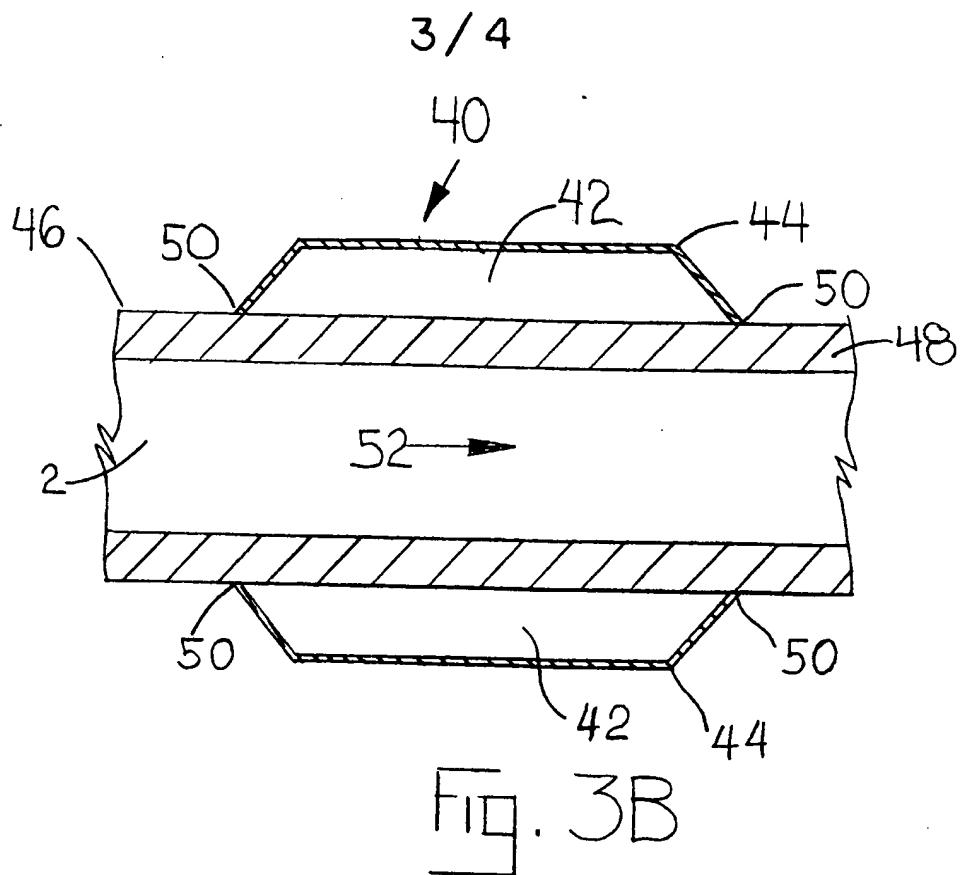
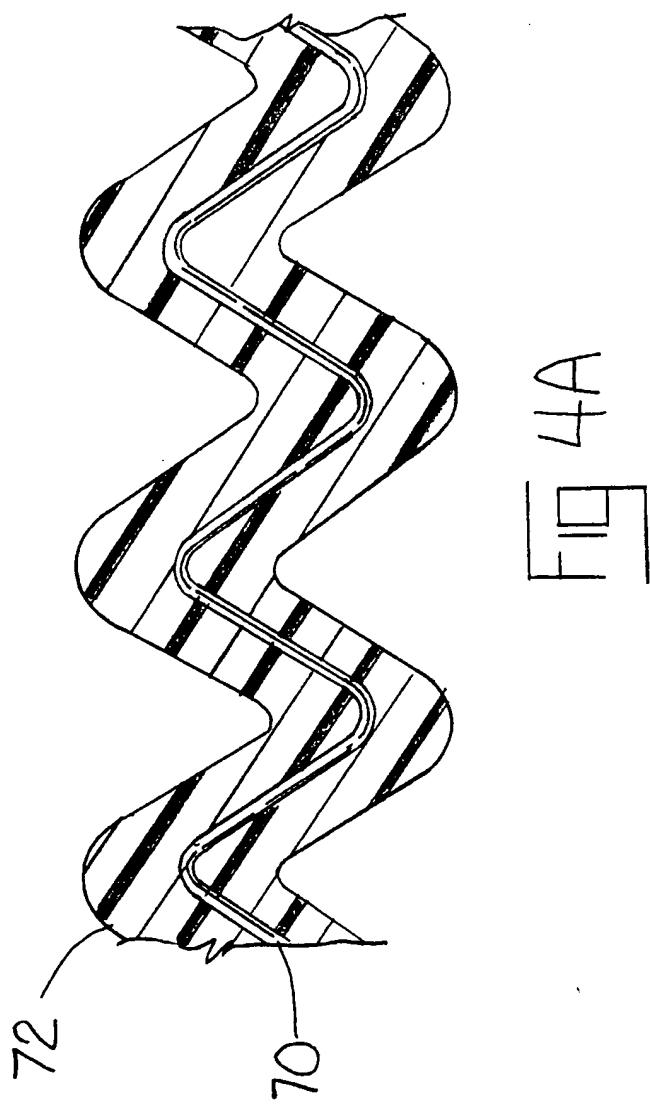


FIG. 2B



4 / 4



-1-

## SEQUENCE LISTING

<110> Ludwig Institute for Cancer Research  
Helsinki University Licensing Ltd. Oy  
Seppo Ylä-Herttuala

<120> Use of VEGF-C or VEGF-D Gene or Protein to Prevent Restenosis

<130> 28967/35601A

<140>

<141>

<150> US 60/105,587  
<151> 1998-10-26

<160> 18

<170> PatentIn Ver. 2.0

<210> 1  
<211> 1997  
<212> DNA  
<213> Homo sapiens

<220>

<221> CDS  
<222> (352)..(1608)

<400> 1  
cccgccccgc ctctccaaaa agctacacccg acgcggaccg cggcggcgctc ctccctcgcc 60  
ctcgcttcac ctcgcgggct ccgaatgcgg ggagctcgga tgtccgggtt cctgtgaggc 120  
ttttacctga caccgcgcgc ctttccccgg cactggctgg gagggcgccc tgcaaagttg 180  
ggaacgcgga gccccggacc cgctccgcgc gcctccggct cgcccagggg gggtcgcggg 240  
gaggagccccg ggggagaggg accaggaggg gcccgccggcc tcgcaggggc gcccgcgccc 300  
ccaccctgc ccccgccagc ggaccggtcc cccaccccg gtccttcac c atg cac 357  
Met His  
1

ttg ctg ggc ttc ttc tct gtg gcg tgt tct ctg ctc gcc gct gcg ctg 405  
Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala Ala Leu  
5 10 15

ctc ccg ggt cct cgc gag gcg ccc gcc gcc gcc ttc gag tcc 453  
Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Phe Glu Ser  
20 25 30

gga ctc gac ctc tcg gac gcg gag ccc gac gcg ggc gag gcc acg gct 501  
Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala Thr Ala  
35 40 45 50

tat gca agc aaa gat ctg gag gag cag tta cgg tct gtg tcc agt gta 549  
Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser Ser Val  
55 60 65

-2-

gat gaa ctc atg act gta ctc tac cca gaa tat tgg aaa atg tac aag	597
Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met Tyr Lys	
70 75 80	
tgt cag cta agg aaa gga ggc tgg caa cat aac aga gaa cag gcc aac	645
Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln Ala Asn	
85 90 95	
ctc aac tca agg aca gaa gag act ata aaa ttt gct gca gca cat tat	693
Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr	
100 105 110	
aat aca gag atc ttg aaa agt att gat aat gag tgg aga aag act caa	741
Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln	
115 120 125 130	
tgc atg cca cgg gag gtg tgt ata gat gtg ggg aag gag ttt gga gtc	789
Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val	
135 140 145	
gcg aca aac acc ttc ttt aaa cct cca tgt gtg tcc gtc tac aga tgt	837
Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys	
150 155 160	
ggg ggt tgc tgc aat agt gag ggg ctg cag tgc atg aac acc agc acg	885
Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr	
165 170 175	
agc tac ctc agc aag acg tta ttt gaa att aca gtg cct ctc tct caa	933
Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln	
180 185 190	
ggc ccc aaa cca gta aca atc agt ttt gcc aat cac act tcc tgc cga	981
Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg	
195 200 205 210	
tgc atg tct aaa ctg gat gtt tac aga caa gtt cat tcc att att aga	1029
Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg	
215 220 225	
cgt tcc ctg cca gca aca cta cca cag tgt cag gca gcg aac aag acc	1077
Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr	
230 235 240	
tgc ccc acc aat tac atg tgg aat aat cac atc tgc aga tgc ctg gct	1125
Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala	
245 250 255	
cag gaa gat ttt atg ttt tcc tcg gat gct gga gat gac tca aca gat	1173
Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp	
260 265 270	
gga ttc cat gac atc tgt gga cca aac aag gag ctg gat gaa gag acc	1221
Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr	
275 280 285 290	
tgt cag tgt gtc tgc aga gca ggg ctt cgg cct gcc agc tgt gga ccc	1269
Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro	
295 300 305	

-3-

cac aaa gaa cta gac aga aac tca tgc cag tgt gtc tgt aaa aac aaa 1317  
 His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys  
 310 315 320  
  
 ctc ttc ccc agc caa tgt ggg gcc aac cga gaa ttt gat gaa aac aca 1365  
 Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr  
 325 330 335  
  
 tgc cag tgt gta tgt aaa aga acc tgc ccc aga aat caa ccc cta aat 1413  
 Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn  
 340 345 350  
  
 cct gga aaa tgt gcc tgt gaa tgt aca gaa agt cca cag aaa tgc ttg 1461  
 Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu  
 355 360 365 370  
  
 tta aaa gga aag aag ttc cac cac caa aca tgc agc tgt tac aga cgg 1509  
 Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg  
 375 380 385  
  
 cca tgt acg aac cgc cag aag gct tgt gag cca gga ttt tca tat agt 1557  
 Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser Tyr Ser  
 390 395 400  
  
 gaa gaa gtg tgt cgt tgt gtc cct tca tat tgg aaa aga cca caa atg 1605  
 Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro Gln Met  
 405 410 415  
  
 agc taagattgtta ctgtttcca gttcatcgat tttctattat ggaaaactgt 1658  
 Ser  
  
 gttgccacag tagaactgtc tgtgaacaga gagacccttg tgggtccatg ctaacaaaga 1718  
 caaaagtctg tctttcctga accatgtgga taactttaca gaaatggact ggagctcatc 1778  
 tgcaaaaggc ctcttgtaaa gactggttt ctgccaatga ccaaacagcc aagattttcc 1838  
 tcttgatt tctttaaaag aatgactata taatttattt ccactaaaaa tattgtttct 1898  
 gcattcattt ttatagcaac aacaattggt aaaactcact gtgatcaata ttttatatc 1958  
 atgcaaaata tgtttaaaat aaaatgaaaa ttgttattat 1997  
  
 <210> 2  
 <211> 419  
 <212> PRT  
 <213> Homo sapiens  
  
 <400> 2  
 Met His Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala  
 1 5 10 15  
  
 Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Phe  
 20 25 30  
  
 Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala  
 35 40 45  
  
 Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser  
 50 55 60

-4-

Ser Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met  
 65 70 75 80  
 Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln  
 85 90 95  
 Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala  
 100 105 110  
 His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys  
 115 120 125  
 Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe  
 130 135 140  
 Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr  
 145 150 155 160  
 Arg Cys Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr  
 165 170 175  
 Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu  
 180 185 190  
 Ser Gln Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser  
 195 200 205  
 Cys Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile  
 210 215 220  
 Ile Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn  
 225 230 235 240  
 Lys Thr Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys  
 245 250 255  
 Leu Ala Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser  
 260 265 270  
 Thr Asp Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu  
 275 280 285  
 Glu Thr Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys  
 290 295 300  
 Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys  
 305 310 315 320  
 Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu  
 325 330 335  
 Asn Thr Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro  
 340 345 350  
 Leu Asn Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys  
 355 360 365  
 Cys Leu Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr  
 370 375 380  
 Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser  
 385 390 395 400

-5-

Tyr	Ser	Glu	Glu	Val	Cys	Arg	Cys	Val	Pro	Ser	Tyr	Trp	Lys	Arg	Pro
405									410					415	

Gln Met Ser

<210> 3  
 <211> 2029  
 <212> DNA  
 <213> Homo sapiens

<220>  
 <221> CDS  
 <222> (411)..(1475)

<400> 3  
 gttgggttcc agctttctgt agctgtaagc attgggtggcc acaccaccc cttacaaagc 60  
 aactagaacc tgcggcatac attggagaga ttttttaat tttctggaca tgaagtaaat 120  
 ttagagtgct ttcttaatttc aggtagaaga catgtccacc ttctgattat ttttggagaa 180  
 cattttgatt ttttcatct ctctctcccc acccctaaga ttgtgcaaaa aaagcgtacc 240  
 ttgcctaatt gaaataattt cattggattt tgatcagaac tgattattt gtttctgtg 300  
 tgaagtttg aggtttcaaa cttccctct ggagaatgcc ttttgaaca atttctcta 360  
 gctgcctgat gtcaactgct tagtaatcag tggatattga aatattcaaa atg tac 416  
 Met Tyr  
 1

aga gag tgg gta gtg aat gtt ttc atg atg ttg tac gtc cag ctg 464  
 Arg Glu Trp Val Val Val Asn Val Phe Met Met Leu Tyr Val Gln Leu  
 5 10 15

gtg cag ggc tcc agt aat gaa cat gga cca gtg aag cga tca tct cag 512  
 Val Gln Gly Ser Ser Asn Glu His Pro Val Lys Arg Ser Ser Gln  
 20 25 30

tcc aca ttg gaa cga tct gaa cag cag atc agg gct gct tct agt ttg 560  
 Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser Ser Leu  
 35 40 45 50

gag gaa cta ctt cga att act cac tct gag gac tgg aag ctg tgg aga 608  
 Glu Glu Leu Ieu Arg Ile Thr His Ser Glu Asp Trp Lys Leu Trp Arg  
 55 60 65

tgc agg ctg agg ctc aaa agt ttt acc agt atg gac tct cgc tca gca 656  
 Cys Arg Leu Arg Ieu Lys Ser Phe Thr Ser Met Asp Ser Arg Ser Ala  
 70 75 80

tcc cat cgg tcc act agg ttt gcg gca act ttc tat gac att gaa aca 704  
 Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Ile Glu Thr  
 85 90 95

cta aaa gtt ata gat gaa gaa tgg caa aga act cag tgc agc cct aga 752  
 Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser Pro Arg  
 100 105 110

-6-

gaa acg tgc gtg gag gtg gcc agt gag ctg ggg aag agt acc aac aca	800
Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Ser Thr Asn Thr	
115 120 125 130	
ttc ttc aag ccc cct tgt gtg aac gtg ttc cga tgt ggt ggc tgt tgc	848
Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly Cys Cys	
135 140 145	
aat gaa gag agc ctt atc tgt atg aac acc agc acc tcg tac att tcc	896
Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser Thr Ser Tyr Ile Ser	
150 155 160	
aaa cag ctc ttt gag ata tca gtg cct ttg aca tca gta cct gaa tta	944
Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro Glu Leu	
165 170 175	
gtg cct gtt aaa gtt gcc aat cat aca ggt tgt aag tgc ttg cca aca	992
Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys Cys Leu Pro Thr	
180 185 190	
gcc ccc cgc cat cca tac tca att atc aga aga tcc atc cag atc cct	1040
Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln Ile Pro	
195 200 205 210	
gaa gaa gat cgc tgt tcc cat tcc aag aaa ctc tgt cct att gac atg	1088
Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys Pro Ile Asp Met	
215 220 225	
cta tgg gat agc aac aaa tgt aaa tgt gtt ttg cag gag gaa aat cca	1136
Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln Glu Asn Pro	
230 235 240	
ctt gct gga aca gaa gac cac tct cat ctc cag gaa cca gct ctc tgt	1184
Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu Pro Ala Leu Cys	
245 250 255	
ggg cca cac atg atg ttt gac gaa gat cgt tgc gag tgt gtc tgt aaa	1232
Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu Cys Val Cys Lys	
260 265 270	
aca cca tgt ccc aaa gat cta atc cag cac ccc aaa aac tgc agt tgc	1280
Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys Ser Cys	
275 280 285 290	
ttt gag tgc aaa gaa agt ctg gag acc tgc tgc cag aag cac aag cta	1328
Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His Lys Leu	
295 300 305	
ttt cac cca gac acc tgc agc tgt gag gac aga tgc ccc ttt cat acc	1376
Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe His Thr	
310 315 320	
aga cca tgt gca agt ggc aaa aca gca tgt gca aag cat tgc cgc ttt	1424
Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys Arg Phe	
325 330 335	
cca aag gag aaa agg gct gcc cag ggg ccc cac agc cga aag aat cct	1472
Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys Asn Pro	
340 345 350	

-7-

tga ttcagcgttc caagttcccc atccctgtca ttttaacag catgctgctt 1525  
 355 tgccaaagttt ctgtcaactgt tttttccca ggtgttaaaa aaaaaatcca ttttacacag 1585  
 caccacagt aatccagacc aaccttccat tcacaccagc taaggagtcc ctgggtcatt 1645  
 gatggatgtc ttctagctgc agatgcctct gcgcaccaag gaatggagag gaggggaccc 1705  
 atgtaatcct tttgttttagt tttgttttg tttttggtg aatgagaaaag gtgtgctggt 1765  
 catggaaatgg caggtgtcat atgactgatt actcagagca gatgaggaaa actgtagtt 1825  
 ctgagtcctt tgctaatcgc aactcttgc aattattctg attctttttt atgcagaatt 1885  
 tgattcgtat gatcagttact gactttctga ttactgtcca gcttatagtc ttccagttta 1945  
 atgaactacc atctgatgtt tcataattaa gtgtattaa agaaaataaa caccattatt 2005  
 caagccaaaa aaaaaaaaaa aaaa 2029

<210> 4  
 <211> 354  
 <212> PRT  
 <213> Homo sapiens

<400> 4  
 Met Tyr Arg Glu Trp Val Val Val Asn Val Phe Met Met Leu Tyr Val  
 1 5 10 15  
 Gln Leu Val Gln Gly Ser Ser Asn Glu His Gly Pro Val Lys Arg Ser  
 20 25 30  
 Ser Gln Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser  
 35 40 45  
 Ser Leu Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp Trp Lys Leu  
 50 55 60  
 Trp Arg Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met Asp Ser Arg  
 65 70 75 80  
 Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Ile  
 85 90 95  
 Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser  
 100 105 110  
 Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Ser Thr  
 115 120 125  
 Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly  
 130 135 140  
 Cys Cys Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser Thr Ser Tyr  
 145 150 155 160  
 Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro  
 165 170 175  
 Glu Leu Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys Cys Leu

-8-

180 185 190

Pro Thr Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln  
 195 200 205

Ile Pro Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys Pro Ile  
 210 215 220

Asp Met Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln Glu Glu  
 225 230 235 240

Asn Pro Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu Pro Ala  
 245 250 255

Leu Cys Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu Cys Val  
 260 265 270

Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys  
 275 280 285

Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His  
 290 295 300

Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe  
 305 310 315 320

His Thr Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys  
 325 330 335

Arg Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys  
 340 345 350

Asn Pro

<210> 5

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 5

ttggaggcct aggcttttgc

20

<210> 6

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 6

atactgtcgt cgtccccctca

20

<210> 7

<211> 22

-9-

<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 7  
ggtagaagac cccaggact tt 22

<210> 8  
<211> 17  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 8  
cgccattcgc cattcag 17

<210> 9  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 9  
ctgcttactg gcttatcg 18

<210> 10  
<211> 20  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 10  
cctgttctct gttatgttgc 20

<210> 11  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 11  
tctccaaaaa gctacacccg 19

<210> 12  
<211> 18  
<212> DNA  
<213> Artificial Sequence

-10-

<220>  
<223> Description of Artificial Sequence: primer  
  
<400> 12  
caagtgc~~at~~tg gtggaagg 18  
  
<210> 13  
<211> 20  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: primer  
  
<400> 13  
tcgatccatg aactttctgc 20  
  
<210> 14  
<211> 20  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: primer  
  
<400> 14  
ttcg~~tta~~aac tcaagctgcc 20  
  
<210> 15  
<211> 19  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: primer  
  
<400> 15  
gaccctggct ttactgctg 19  
  
<210> 16  
<211> 20  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: primer  
  
<400> 16  
ggaacattta cacgtctg~~cg~~ 20  
  
<210> 17  
<211> 2679  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: chimeric

-11-

sequence in which CMV promoter sequence is ligated  
to Homo sapien VEGF-C sequence

<400> 17  
cgttgacatt gattattgac tagttattaa tagtaatcaa ttacgggtc attagttcat 60  
agcccatata tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg 120  
cccaacgacc cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata 180  
gggactttcc attgacgtca atgggtggac tatttacggt aaactgccc cttggcagta 240  
catcaagtgt atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc 300  
gcctggcatt atgcccagta catgacctta tgggacttgc ctacttggca gtacatctac 360  
gtattagtca tcgcttattac catggtgatg cggtttggc agtacatcaa tggcgtgga 420  
tagcggtttgc actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttgc 480  
ttttggcacc aaaatcaacg ggactttcca aatgtcgta acaactccgc cccattgacg 540  
caaatggcg gttaggcgtgt acggtggag gtctatataa gcagagctct ctggctaact 600  
agagaacccca ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa 660  
gctggctagc gtttaaactt aacccggccc gcctctccaa aaagctacac cgacgcggac 720  
cgccggggcg tcctccctcg ccctcgcttc acctcgccgg ctccgaatgc gggagctcg 780  
gatgtccgggt ttccctgtgag gcttttacct gacacccgccc gcctttccccc ggcactggct 840  
gggagggcgcc cctgcaaagt tgggaacgcg gagcccccga cccgctcccg ccgcctccgg 900  
ctcgccccagg ggggtcgcc gggaggagcc cgggggagag ggaccaggag gggcccgccg 960  
cctcgccagg gcgcggcgcc ccccacccct gccccggcca gcggaccggc ccccacccca 1020  
cggtccctcc accatgcact tgctggctt ctctctgtg gcgtgttctc tgctcgccgc 1080  
tgcgctgctc cgggtcctc gcgaggcgcc cgccgcccgc gccgccttcg agtccggact 1140  
cgacctctcg gacgcggagc cgcacgcggg cgaggccacg gcttatgcaa gcaaagatct 1200  
ggaggagcag ttacggctcg tgcgtgttgc agatgaactc atgactgtac tctacccaga 1260  
atattggaaa atgtacaagt gtcagctaag gaaaggaggc tggcaacata acagagaaca 1320  
ggccaacctc aactcaagga cagaagagac tataaaattt gctgcagcac attataatac 1380  
agagatcttgc aaaagtattt gataatgatg gagaagact caatgcattc cacgggaggt 1440  
gtgtatagat gtggggagg agtttggagt cgcgacaaac accttcttta aacctccatg 1500  
tgtgtccgtc tacagatgtg ggggttgctg caatagttag gggctgcagt gcatgaacac 1560  
cagcacgagc tacctcagca agacgttatt tggaaattaca gtgcctctc ctcaaggccc 1620  
caaaccagta acaatcagtt ttgccaatca cacttcctgc cgatgcattt ctaaactggaa 1680  
tggttacaga caagttcatt ccattattag acgttccctg ccagcaacac taccacagtg 1740

-12-

tcaggcagcg aacaagacct gccccaccaa ttacatgtgg aataatcaca tctgcagatg 1800  
cctggctcag gaagattta tgtttcctc ggatgctgga gatgactcaa cagatggatt 1860  
ccatgacatc tgtggaccaa acaaggagct ggatgaagag acctgtcagt gtgtctgcag 1920  
agcggggctt cggcctgcca gctgtggacc ccacaaagaa ctagacagaaa actcatgcca 1980  
gtgtgtctgt aaaaacaaac tcttccccag ccaatgtggg gccaaccgag aatttcatg 2040  
aaacacatgc cagtggttat gtaaaagaac ctgccccaga aatcaacccc taaatcctgg 2100  
aaaatgtgcc tgtgaatgta cagaaagtcc acagaaatgc ttgttaaaag gaaagaagtt 2160  
ccaccaccaa acatgcagct gttacagacg gccatgtacg aaccgcgcaga aggcttgtga 2220  
gccaggattt tcatatagtg aagaagtgtg tcgttgtgtc ccttcatatt ggaaaagacc 2280  
acaaatgagc taagattgta ctgtttcca gttcatcgat tttctattat ggaaaactgt 2340  
gttgcacacag tagaactgtc tgtgaacaga gagacccttg tgggtccatg ctaacaaaga 2400  
caaaaagtctg tcttcctga accatgtgga taactttaca gaaatggact ggagctcatc 2460  
tgcaaaaaggc ctcttgtaaa gactggttt ctgccaatga ccaaacagcc aagattttcc 2520  
tcttgtgatt tctttaaaag aatgactata taatttattt ccactaaaaa tattgtttct 2580  
gcattcattt ttatagcaac aacaattggt aaaactcaact gtgatcaata ttttatatc 2640  
atgc当地 aataatgaaaa ttgttattat 2679

<210> 18  
<211> 2240  
<212> DNA  
<213> *Mus musculus*

<400> 18  
tggtagaag atgaaccgta agcctaggct agaactgagg gaggctacta ctcccaccct 60  
tccgagggtt ggccgcagga ctgggcagct ggcctaccta ctttctgaa tgctaggta 120  
gtttgaatc accatgccgg cctggcccg cttctgcccc attggcaccc tggcttcagt 180  
tccctggcaa catctctgtg tgtgtgtgtg tgtgtgagag agagagatca ggaggaacaa 240  
gggcctctgt ctgcccagca gttgtctctc cttcaggct ctgccagact acacagtgc 300  
tacgtgggtt tccacaggtc gtctcactcc ccgcactga ctaactccag aactccaccc 360  
ccgttctcag tgccacaaat ttggtgccaa attctctcca gagaagcctc tctggaaact 420  
tcccagagga tcccattcac cccagggccc tagctctga tgactgcaga tcagacaagg 480  
gctcagataa gcatactccc cccccccgt aaccctctcc ccacatataa acctacagtt 540  
atgcttccga ggtcaaacac gcaactttt gggtgtgtgt gtatgtcaga aacacgcaat 600  
tatggggag ctcaaagtct gccgcactca agaatcatct ctcacccct ttccaagacc 660  
cgtgccattt gagcaagagt tgggtgtgc ataatgttagt cactaggggg cgctcggcca 720

-13-

tcacggggag atcgtaactt gggcgagccg agtctgcgtg agggaggacg cgtgtttcaa 780  
tgtgagtgcg tgcgtgcgtgt gtgtgtgtgt gtgtgtgtg tggaggtgg gggagaaagc 840  
caggggtcac tctagttgtc cctatcctca tacgttcctg ccagctctcc gccttccaac 900  
ccctactttc tcctatatcc tggaaagg aattgtctta gaccctgtcc gcatataacc 960  
tcactctcct gtctccctg attccaaata ctctggatt cccagtgtgt tcctgagccc 1020  
atttgaaggg gtgcacagat aattttgagg ccgtggaccc tggtaagggg tttagcttc 1080  
catttcgcgg tagtggccta gggctcccc gaaaggcggt gcctggctcc accagaccgt 1140  
ccccggggcg ggtctggcg gggctttttt gtggagctag atttcctt tttttccac 1200  
cgctgttacc ggtgagaagc gcagaggctt gggcagccg agctgcagcg agcgcgcggc 1260  
actggggcg agctgagcgg cggcagcgg aactctgtcgc gagacgcagc gacaaggcag 1320  
actatcagcg gactcaccag cccggagtc tggctctgg gatttgatat tcaaacctct 1380  
taattttttt ttcttaaact gtattttttt acgtttaat ttatttttgc ttccttattcc 1440  
cctcttaaat cgtgccaacg gtttggggag gttgggttctt cactccctca aatcaacttcg 1500  
gattgtggaa atcagcagac gaaagaggtt tcaagagctc cagagagaag tcaaggaaga 1560  
gagagagaga ccggtcagag agagcgcgtt ggcgagcga cagagagagg gacagggca 1620  
aagttgactt gaccttgctt ttgggggtga ccggcagagc gggcggtgac ctcccccttc 1680  
gatcttgcat cggaccagtc ggcgtgacgg acagacagac agacaccgc cccagcccc 1740  
gcgcacccaccc cctcgccggc gggctgccga cgggtggacgc ggcggcgagc cgagaaaccc 1800  
aagcccgccg cccggaggccg gtggaggggg tcggggctcg cgggattgca cggaaacttt 1860  
tcgtccaaact tctgggtctt tctcgctccg tagtagccgt ggtctgcgcc gcaggagaca 1920  
aaccgatccg gagctggag aaggctagct cggcccttgg gaggccgggg cccgagaaga 1980  
gaggggagga aggaagagga gagggggcca cagtgccgc tcggctctca ggagccgagc 2040  
tcatggacgg gtgaggccgc cgtgtgcgc gacagtgcgc cagccgcgc cgcgcggcc 2100  
gccccggccc gggcctcggt tccagaaggg agaggagccc gccaaggcgc gcaagagagc 2160  
gggctgcctc gcagttccgga gccggagaga gggagcgcga gccgcccggg cccggacgg 2220  
cctccgaaac catgaacttt 2240